Contribution of Synaptic Disinhibition to Central Sensitization in Migraine Pain

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2012

Ai Miei Nonni

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Summary

Migraine is a common and disabling neurovascular disorder of still poorly understood etiology. Migraine patients suffer from episodic events of unilateral headache often accompanied by nausea, photophobia and phonophobia. A better understanding of the neurobiological mechanisms of migraine is needed for the development of new therapeutic approaches. While general consensus exists on the importance of the involvement of the trigeminovascular system in migraine headache, the mechanisms leading to central trigeminal sensitization are not known. At least in a subgroup of migraine patients, cortical spreading depression is an event that precedes migraine headache and trigeminal sensitization.

In this thesis, I have addressed the hypothesis that diminished synaptic inhibition occurs at the level of the brainstem as a consequence of cortical spreading depression and contributes to pain during migraine attacks. The presence of cutaneous allodynia (i.e. a pathologically increased sensitivity to light touch) points to diminished glycinergic inhibitory control and its reversal by cyclooxygenase inhibitors to a contribution of prostaglandins. I therefore specifically addressed whether the previously described prostaglandin E_2 (PGE_2)-mediated inhibition of glycine receptors contributes to central sensitization in migraine. This pathway has previously been shown to make a major contribution to inflammation-induced central pain sensitization in response to peripheral inflammation.

Here, I have used *cacna1a* (R192Q) point mutated mice as a genetic mouse model of migraine and mice deficient in EP2 receptors ($EP2^{-/-}$ mice) or in the glycine receptor α3 subunit ($GlyR\alpha3^{-/-}$ mice), which both lack inflammation induced central pain sensitization. I compared mice carrying one of these genetic mutations and double mutant mice with corresponding wild-type mice in several behavioral, electrophysiological and neurochemical experiments. In electrophysiological experiments, c*acna1a* (R192Q) mutant mice displayed increased susceptibility to chemically evoked cortical spreading depression but did not show signs of spontaneously cortical spreading depression in long-term EEG recordings. They did not exhibit increased aversion to light and c-*fos* expressions measured in naïve mice or after

chemically induced cortical spreading depression were not different from those of wild-type mice. Photophobia and c-*fos* expression in EP2$^{-/-}$ and GlyRα3$^{-/-}$ mice were also not different from wild-type mice, however differences were found in chemically-induced cortical spreading depression with GlyRα3$^{-/-}$ mice behaving like wild-type mice and EP2$^{-/-}$ mice showing a susceptibility comparable to that of *cacna1a* (R192Q) mice. The latter finding was replicated with an EP2 receptor antagonist and may be relevant with respect to chronic headaches seen during prolonged abuse of cyclooxygenase inhibitors.

Although these findings do not support a major contribution of the EP2/GlyRα3 pathway to pain sensitization in migraine, many lines of evidence still suggest that an altered excitation/inhibition balance is a major factor contributing to migraine pathophysiology. I therefore characterized in a separate project the antihyperalgesic effect of HZ166, a new partial BDZ-site agonist with preferential activity at α2 and α3 $GABA_A$ receptors. Previous experiments in genetically modified mice have suggested that a facilitation of these two $GABA_A$ receptor subtypes can compensate for neuropathy and inflammation-induced losses in inhibitory synaptic pain control. HZ166 dose-dependently reversed signs of hyperalgesia and allodynia associated with neuropathic and inflammatory pain without causing sedation. This compound may therefore be used for future proof-of-concept studies addressing the possibility that drugs enhancing synaptic inhibition could be used to prevent central pain sensitization in migraine.

Riassunto

L'emicrania é una patologia neurovascolare cronica molto comune le cui cause sono ad oggi poco chiare. Un tipico attacco di emicrania è caratterizzato da mal di testa unilaterale accompagnato da tipici sintomi quali nausea, fotofobia e fonofobia. Una migliore comprensione dei meccanismi alla base di questa patologia è di primaria importanza al fine di sviluppare nuove strategie terapeutiche. Mentre esiste un consenso generale in merito all'importanza del sistema trigemino-vascolare, i meccanismi che causano la sensitizzazione centrale ad oggi non sono noti. Almeno in un sottogruppo di pazienti la *cortical spreading depression* (CSD) è un evento che precede la cefalea e la sensitizzazione del sistema trigemino-vascolare. In questa tesi, ho esaminato l'ipotesi che una ridotta inibizione della trasmissione sinaptica si possa verificare a livello del midollo allungato come conseguenza della CSD e possa contribuire alla cefalea durante un attacco di emicrania. La presenza di allodinia cutanea (aumentata sensibilità a uno stimolo normalmente innocuo, che invece viene percepito come doloroso) indica il coinvolgimento di una diminuizione della trasmissione glicinergica ed il fatto che questa allodinia venga curata con successo mediante la somministrazione di inibitori delle cicloossigenasi suggerisce il contributo dei della via del segnale delle cicloossigenasi. A tal proposito, la mia analisi si è concentrata sul ruolo della inibizione glicinergica causata dall'attivazione del recettore EP2 e sull'ipotesi che questa attivazione possa causare sensitizzazione centrale. Secondo precedenti studi, questo pathway è coinvolto nei meccanismi che causano la sensitizzazione centrale che si sviluppa a causa di un'infiammazione periferica e che è coinvolta nel fenomeno del dolore a essa associato. In questa tesi ho utilizzato topi *knock-in* portanti la mutazione R192Q nel gene *cacna1a* come modello genetico di emicrania e topi *knock-out* per il recettore EP2 o per il recettore glicinergico del sottotipo α3 (GlyRα3), entrambi caratterizzati dal mancato sviluppo di sensitizzazione centrale in seguito a infiammazione periferica. Ho confrontato questi topi con topi portanti entrambe le mutazioni e con topi *wild-type* usando diversi approcci sperimentali, in particolare quelli elettrofisiologico, comportamentale e neurochimico. I topi portanti la mutazione R192Q sono risultati piu suscettibili allo sviluppo

di CSD ma non hanno mostrato segni di CSD spontanee in registrazioni EEG. Inoltre, rispetto ai corrispettivi *wild-type*, non hanno manifestato un'aumentata avversione alla luce o un maggior numero di cellule positive per c-*fos* in condizioni basali o in seguito all'induzione di CSD. Simili risultati sono stati ottenuti con i topi *knock-out* per il recettore EP2 e per il recettore GlyRα3. Tuttavia, mentre topi *knock-out* per il recettore GlyRα3 hanno presentato un simile livello di suscettibilità all'induzione di CSD confrontati con i topi *wild-type*, i topi *knock-out* per il recettore EP2 hanno mostrato valori simili a quelli ottenuti in topi mutant *cacna1a* (R192Q). Questi risultati sono stati in seguito replicati con l'uso di un antagonista del recettore EP2 e potrebbero essere importanti per spiegare il mal di testa cronico causato dall'uso improprio di inibitori delle ciclossigenasi. Nonostante i nostri risultati non abbiano evidenziato un maggiore contributo del pathway EP2/GlyRα3 alla sensitizzazione centrale associata alla patogenesi dell'emicrania, alcune linee di ricerca suggeriscono che un alterato equilibrio fra eccitazione ed inibizione è un processo chiave del comportamento dei circuiti neuronali corticali e potrebbe essere determinante nella patogenesi dell'emicrania. A tal proposito ho caratterizzato un nuovo composto, HZ166, un agonista al sito di legame delle benzodiazepine con attività preferenziale verso i sottotipi recettoriali α2 e α3 dei recettori $GABA_A$. Studi effettuati precedentemente dal nostro gruppo in modelli murini geneticamente modificati hanno mostrato che una facilitazione farmacologica di questi due recettori può compensare la perdita di inibizione sinaptica tipica di forme di dolore cronico quali neuropatie e dolore infiammatorio. HZ166 è risultato efficace nel mitigare segni di iperalgesia e allodinia associati con situazioni di neuropatia e infiammazione senza provocare sedazione nell'animale. Questo composto potrebbe essere utilizzato nel futuro in studi di *proof- of-concept* volti a determinare se farmaci che aumentino la trasmissione sinaptica inibitoria possano essere utilizzati per prevenire la sensitizzazione centrale associata alla patogenesi dell'emicrania.

Zusammenfassung

Migräne ist eine häufige und entkräftende neurovaskuläre Störung mit einer immer noch schlecht verstanden Ätiologie. Die Patienten leiden unter (episodischen) wiederkehrenden Ereignissen einseitigen Kopfschmerzes, der oft mit Übelkeit, Photophobie und Phonophobie einhergeht. Ein besseres Verständnis der der Migräne zugrunde liegenden neurobiologischen Mechanismen ist für die Entwicklung neuer therapeutischer Ansätze erforderlich. Während ein allgemeiner Konsens über die Bedeutung der Beteiligung des trigeminovaskulären Systems für die Migräne existiert, sind die Mechanismen, die eine zentralen Trigeminus- Sensibilisierung bewirken, nicht bekannt. Zumindest in einer Untergruppe der Migräne-Patienten ist „Cortical Spreading Depression (CSD)“ ein Ereignis, welches der Migräne und der Trigeminus- Sensibilisierung vorangeht. In meiner Doktorarbeit habe ich die Hypothese, dass eine verminderte synaptische Hemmung auf der Ebene des Hirnstamms als Folge von CSD auftritt, und zu den Schmerzen während der Migräne-Attacken beiträgt. Das Auftreten von kutaner Allodynie (d.h. eine krankhaft erhöhte Empfindlichkeit gegenüber leichten Berührungen) deutet auf eine verminderte glycinerge inhibitorische Kontrolle und ihre Umkehrung durch Cyclooxygenase-Inhibitoren auf einen Beitrag des Cyclooxygenase Signalwegs hin. Ich habe daher gezielt untersucht, ob PGE_2-vermittelte Hemmung von Glycin-Rezeptoren (Harvey et al., 2004) eine zentrale Sensibilisierung bei Migräne hervorruft. Für diesen Signalweg ist bereits gezeigt worden, dass er einen wichtigen Beitrag zur entzündungsbedingten zentralen Schmerzsensibilisierung leistet.

Hier habe ich *cacna1a* (R192Q) punktmutierte Mäuse als genetisches Mausmodell der Migräne verwendet, und auch Mäuse mit „loss-of-function“ Mutationen in den EP2-Rezeptoren ($EP2^{-/-}$ Mäuse) oder in der Glycin-Rezeptor-Untereinheit α3 ($GlyR\alpha3^{-/-}$ Mäuse), welchen beiden die entzündungsinduzierte zentrale Schmerzsensibilisierung fehlt. Ich verglich Mäuse mit jeweils einer dieser genetischen Mutationen sowie doppelt mutierte Mäuse mit den entsprechenden Wildtyp-Mäusen in verschiedenen verhaltens-, elektrophysiologischen und neurochemischen Experimenten. In elektrophysiologischen Experimenten zeigten *cacna1a* (R192Q) mutierte Mäuse eine erhöhte Anfälligkeit für

chemisch hervorgerufene CSD, aber keine Zeichen von spontaner CSDs in Langzeit-EEG-Aufnahmen.

Sie zeigten keine erhöhte Abneigung gegen Licht, und C-fos-Expression, die in unbehandelten Mäusen oder nach chemisch induzierter CSD gemessen wurde, unterschied sich nicht von jener in Wildtyp-Mäusen. Photophobie und C-fos-Expression in $EP2^{-/-}$ und $GlyR\alpha3^{-/-}$ Mäuse waren auch nicht von Wildtyp-Mäusen unterscheidbar, jedoch wurden Unterschiede in chemisch induzierter CSD gefunden: $GlyR\alpha3^{-/-}$ Mäuse verhalten sich wie Wildtyp-Mäuse, und $EP2^{-/-}$ Mäuse zeigten eine Empfindlichkeit vergleichbar mit der der *cacna1a* (R192Q) Mäuse. Letzteres wurde mit einem EP2 -Rezeptor-Antagonist repliziert und könnte in Bezug auf chronische Kopfschmerzen während des Missbrauchs von Cyclooxygenase-Inhibitoren relevant sein.

Obwohl diese Ergebnisse keinen wesentlichen Beitrag des EP2/GlyRα3 Signalwegs zur Schmerzsensibilisierung bei Migräne unterstützen, weisen viele Beweislinien immer noch darauf hin, dass ein verändertes Gleichgewicht von Erregung und Hemmung ein wichtiger Faktor in der Pathophysiologie der Migräne ist. Daher habe ich in einem separaten Projekt die antihyperalgetische Wirkung von HZ166 (eines neue partiellen BDZ-Site Agonisten mit verstärkter Aktivität an α2 und α3 $GABA_A$-Rezeptoren) charakterisiert.

Frühere Experimente mit gentechnisch veränderten Mäusen deuten darauf hin, dass eine Aktivierung dieser beiden $GABA_A$-Rezeptor-Subtypen den durch Neuropathie und Entzündung bedingten Verlust der hemmenden synaptischen Schmerzkontrolle kompensieren kann. HZ166 kehrt, ohne eine Sedierung zu bewirken, in einer dosisabhängigen Weise die Zeichen von Hyperalgesie und Allodynie um, die mit neuropathischen und entzündlichen Schmerzen in Verbindung stehen. Diese Substanz kann daher für zukünftige „Proof-of-Concept“ Studien verwendet werden. Diese Studien werden sich mit der Möglichkeit befassen, dass Medikamente, welche die synaptische Inhibition verbessen, dazu benutzen werden könnten, die zentrale Schmerzsensibilisierung bei Migräne zu verhindern.

GENERAL INTRODUCTION

Definition and epidemiology

Migraine is a common episodic neurovascular disorder characterized by attacks of severe headache and a variety of other neurological symptoms. It affects about 50 million people within the European Union with a prevalence of 6-8% in men and 15-25 % in women (Stovner *et al.*, 2006). In addition, it costs the European Union more than €27 billion per year (Olesen *et al.*, 2012a; Woods *et al.*, 1994). Diagnosis of migraine is typically achieved according to well-accepted international criteria, which mainly rely on the patient's description of symptoms and on the exclusion of headaches secondary to other diseases. The frequency of migraine attacks distinguishes episodic migraine (migraine headache on 1-14 days/month for >3 months) from chronic migraine (migraine headache on >15 days/month for >3 months) (International Headache Society Headache Classification Committee, 2004).

A typical migraine headache usually lasts between 4 and 72 hours and is unilateral, throbbing and aggravated by physical activity. The headache is often accompanied by other symptoms, such as nausea, vomiting and hypersensitivity to light (photophobia) and/or sound (phonophobia). In one third of patients, the headache is usually preceded by an aura, which manifests in focal neurological symptoms that develop gradually within 5-20 minutes and last less than 60 minutes. The presence or absence of the aura allows the distinction of two different types of migraine: migraine without aura (MO) and migraine with aura (MA). Aura symptoms are most commonly visual, including the classical scintillating scotoma (99% of patients), sensory disturbances (paraesthesia, 31%), speech difficulties (dysarthria or aphasia, 18%) and motor symptoms (paresis, 6%) (Pietrobon *et al.*, 2003). In about 60% of migraines, the headache is preceded by a premonitory phase consisting of vague neurological changes, the so called prodromal or premonitory symptoms which can forewarn of a migraine attack (International Headache Society Headache Classification Committee, 2004). The most common prodromal symptoms reported in clinical studies are yawning, sleepiness and dysphoria. Other phenomena described in the premonitory phase include altered mood, irritability, depression or euphoria, fatigue, craving for certain foods, stiff muscles, constipation or diarrhea, and hypersensitivity to odors or noise (Buzzi *et al.*, 2005; Rossi *et al.*, 2005).

Migraine patients, especially those suffering from migraine with aura, have an increased risk of other episodic brain disorders, so called comorbidities (Scher *et al.*, 2005). The most common and significant risks are for epilepsy (Le *et al.*, 2011), depression and anxiety disorders (McWilliams *et al.*, 2004), and stroke (Kurth *et al.*, 2005). The association between migraine and epilepsy is especially evident for familial hemiplegic migraine (FHM). Both diseases are considered to be disorders involving neuronal hyperexcitability and dysfunctional ion transport (Scher *et al.*, 2005).

Risk factors and trigger factors

Because migraine is a chronic episodic disorder it is important to distinguish between risk factors for the disorder and triggering factors for individual attacks. Examples of these risk factors include family history, genetics, gender, and incidence of epilepsy and/or depression (Lipton, 2000). A trigger for migraine is any factor that on exposure or withdrawal leads to the development of a migraine attack. Putative migraine triggers include a variety of endogenous (e.g., menses) and exogenous (e.g. diet, medication withdrawal, weather changes, stress) factors. Among these, stress is the most important trigger for migraine in both males and females (Martin, 2010). In some patients, the occurrence of migraine is also often preceded by the intake of certain food products such as alcohol (including wine), cheese, chocolate as well as withdrawal of caffeine and “fasting” (Chabriat *et al.*, 1999). In addition, clinical evidence indicates a strong association between female sex hormones and the occurrence of migraine attacks. Indeed, menstruation is an important trigger for female headache sufferers and it has been shown that there is a decrease in migraine frequency during pregnancy (MacGregor, 2004).

Pharmacological management of migraine

Acute treatment

Non-steroidal anti-inflammatory drugs (NSAIDs) have been used in the treatment of acute migraine attacks for the last 40 years (Burstein *et al.*, 2011). They act through the inhibition of the enzymes cyclooxygenase (COX) 1 and 2, causing a decrease in the synthesis of prostaglandins from arachidonic acid (Williams, 2005). While COX-1 is constitutively expressed and produces prostaglandins involved mainly in gastrointestinal mucosal protection, COX-2, the inducible form, generates prostaglandins in response to inflammatory stimuli (Vane *et al.*, 1998). Most of NSAIDs act as inhibitors of both COX isoforms. NSAIDs have been extensively studied in clinical trials, where they have been shown to be superior to placebo and to display a comparable efficacy to other acute migraine treatments such as triptans (Rasmussen *et al.*, 2001). According to recent meta-analyses, aspirin and NSAIDs, such as naproxen and ibuprofen, have an efficacy in acute migraine that is close to that of oral sumatriptan (Magis *et al.*, 2011). A more detailed description of their mechanisms of action is provided in "Peripheral and central sensitization".

Other options exist for managing acute migraine attacks. Triptans are presently the most important anti-migraine drugs. Sumatriptan was the first member of the triptans to be described and is the most extensively studied migraine medication (Ferrari *et al.*, 2001; Humphrey *et al.*, 1991b). Triptans are not general analgesics but are effective against neurovascular forms of headache (migraine and cluster headache) (Ashkenazi *et al.*, 2011), and a large number of high quality clinical trials have confirmed their efficacy (Ferrari *et al.*, 2001). They exert their effect through specific mechanisms at the neurovascular level (Humphrey *et al.*, 1991a). Triptans are agonists of serotonin receptors $5HT_{1B}$, $_{1D}$ and $_{1F}$. Most available triptans are highly selective for the $5HT_{1B}$ and $5HT_{1D}$ receptor subtypes. While the triptan-mediated activation of $5HT_{1B}$ receptors induces vasoconstriction of the cranial vasculature, activation of $5HT_{1D}$ located at the peripheral and central ends of the sensory trigeminal neurons mediates hyperpolarization of the nerve terminals, which results in an inhibition of the trigeminal excitation and transmission of signals to central neurons (Pytliak *et al.*, 2011). The triptans also inhibit the release of inflammatory peptides in the meninges

and thereby interfere with the transduction of pain signals to the trigeminal nucleus caudalis (TNC) (Dahlof, 2002; Dodick *et al.*, 2004). Although triptans are currently the drugs of choice for acute migraine treatment, they still have some drawbacks: incomplete and inconsistent pain relief (in approximately a third of patients), the recurrence of the headache and failure to alleviate headache when taken during the aura or premonitory phase (Johnston *et al.*, 2010). Triptan-specific side-effects and cardiovascular safety are also major issues for an unsatisfactory performance of this quite recent class of anti-migraine drugs (Goadsby, 2010).

Antiemetic drugs are also used as treatments for acute migraine attacks either alone or in combination with other analgesics (International Headache Society Headache Classification Committee, 2004). Since many migraine patients require specific treatment for the nausea and vomiting accompanying their migraine attacks, the addition of an antiemetic can improve outcomes not only by alleviating nausea and vomiting but also by enhancing the bioavailability of the triptans or COX inhibitors (Azzopardi *et al.*, 2008). Antiemetic drugs alone have been shown to be effective as a treatment for acute migraine attacks when administered parentally. Intravenous metoclopramide, an antagonist of the D2 dopamine receptor in the brainstem chemoreceptor trigger zone, has demonstrated a similar efficacy in reducing pain in severe migraine as sumatriptan and opioids (Friedman *et al.*, 2005). Moreover, intravenous administration of chlorpromazine, another D2 receptor antagonist, was also highly effective in treating nausea, photophobia, phonophobia and aura (Bigal *et al.*, 2002). While different studies suggest an anti-migraine effect of parentally administered antiemetics, no evidence supports migraine-specific effects of oral antiemetics, other than relieving nausea (Gilmore *et al.*, 2011).

Prophylactic treatment

Despite our still limited knowledge of the neurobiology of migraine, preventive medications have been successfully used for reducing the incidence and the severity of migraine attacks. Amongst these drugs, anticonvulsants have been effectively employed for migraine prophylaxis. The rationale behind the use of antiepileptic drugs in migraine involves the hypothesis that migraine and epilepsy share several pathogenic mechanisms (Welch, 2005). Indeed, anticonvulsants have been shown to significantly reduce migraine frequency when

compared with placebo (Mulleners *et al.*, 2008). Several effects of the antiepileptic drugs may be responsible for their anti-migraine action, such as increased release of the inhibitory transmitter GABA, antagonism of the excitatory transmitter glutamate and inhibition of sodium channels. Topiramate is probably the best documented migraine prophylactic drug in both episodic and chronic migraine. It is a broad-spectrum antiepileptic drug that is effective for treatment of many types of seizure in adults and children. Although the exact mechanism of action is not known, its anti-migraine efficacy could be due to the blockade of voltage-dependent sodium channels, potentiation of GABA-mediated neurotransmission, antagonism of AMPA/kainate glutamate receptors and inhibition of the carbonic anhydrase enzyme (Edvinsson *et al.*, 2010). Other anticonvulsants reported as prophylactic drugs include valproate, lamotrigine and gabapentin (Bianchin *et al.*, 2010). In particular tonabersat, a blocker of gap junctions, exhibits anticonvulsive properties in experimental animal models (Garza, 2010). Preliminary clinical studies showed that tonabersat is highly efficacious in relieving headache in patients suffering from MA but not in MO (Hauge *et al.*, 2009). If larger studies confirm these findings, tonabersat may be the first drug with a selective effect on MA.

Another drug with accepted efficacy in migraine prophylaxis is the monoamine reuptake inhibitor amitriptyline. Although antidepressants in general share comparable efficacy for the treatment of depressive disorders, their efficacy in migraine prevention varies widely (Smitherman *et al.*, 2011). A recent meta-analysis suggests that tricyclic antidepressants are superior to selective serotonin re-uptake inhibitors for migraine prophylaxis (Jackson *et al.*, 2010).

β-blockers have also been widely used for the prevention of migraine. Their mechanism of action is not known, but it is almost certainly not related to a decrease in blood pressure. Inhibition of β1-mediated effects could be considered the main mechanism of action (Hanbauer *et al.*, 1975). Propranolol and metoprolol have been evaluated in randomized placebo controlled trials for their efficacy in migraine prophylaxis. Propranolol is effective at preventing migraines in the short term, while insufficient data exists to support a conclusion on long term treatment (Linde et al., 2004). β-blockers represent a good choice of preventive drug for migraine patients with preexisting hypertension.

Additionally, calcium channel blockers are well characterized for preventing migraine attacks. They act mainly through the blockade of L-type channels including those expressed in the trigeminal ganglion (Kim *et al.*, 1999). Flunarazine has been shown to reduce the frequency of migraine attacks (Reveiz-Herault *et al.*, 2003), although the mechanism of action remains unclear. It has been proposed that flunarazine could act against cerebral hypoxia by inhibiting the contraction of vascular muscles (Galletti *et al.*, 2009). Calcium channel blockers that are more widely available, such as amlodipine and verapamil, are effective options for some patients (Dandapani *et al.*, 1998; Yu *et al.*, 2003).

Methysergide, introduced as an anti-migraine drug more than 50 years ago, is still considered to be effective in preventing migraine. Its therapeutic effect in migraine prophylaxis has been associated with its antagonism of the 5-HT_{2B} receptor. However, because of severe side-effects, it should be reserved for severe cases in which other migraine preventive drugs are not effective (Koehler *et al.*, 2008).

Medication over-use headache

Medication-overuse headache (MOH) is a form of chronic migraine caused by overuse of antimigraine drugs. MOH manifests as increased frequency and intensity of migraine attacks and enhanced sensitivity to stimuli that elicit migraine episodes. It is characterized by attacks occurring on more than 14 days per month for at least three months (Olesen *et al.*, 2006). It has been proposed that repeated use of antimigraine drugs may cause an increase in the frequency of attacks as a consequence of plastic changes in neurons that alter the responsiveness to migraine triggers (Bigal *et al.*, 2008). All known treatments taken for symptomatic headache relief, including triptans, analgesics, and opioids, can induce transformation from episodic headache to MOH. Interestingly, the withdrawal of medication often results in a dramatic improvement (i.e. reduction) of headache frequency. There is considerable variability in the ability of migraine treatments to lead to development of MOH. Overuse of NSAIDs, for example, has been shown to be associated with MOH in certain patients (Limmroth *et al.*, 2002; Starling *et al.*, 2011) .

Neurobiology of migraine

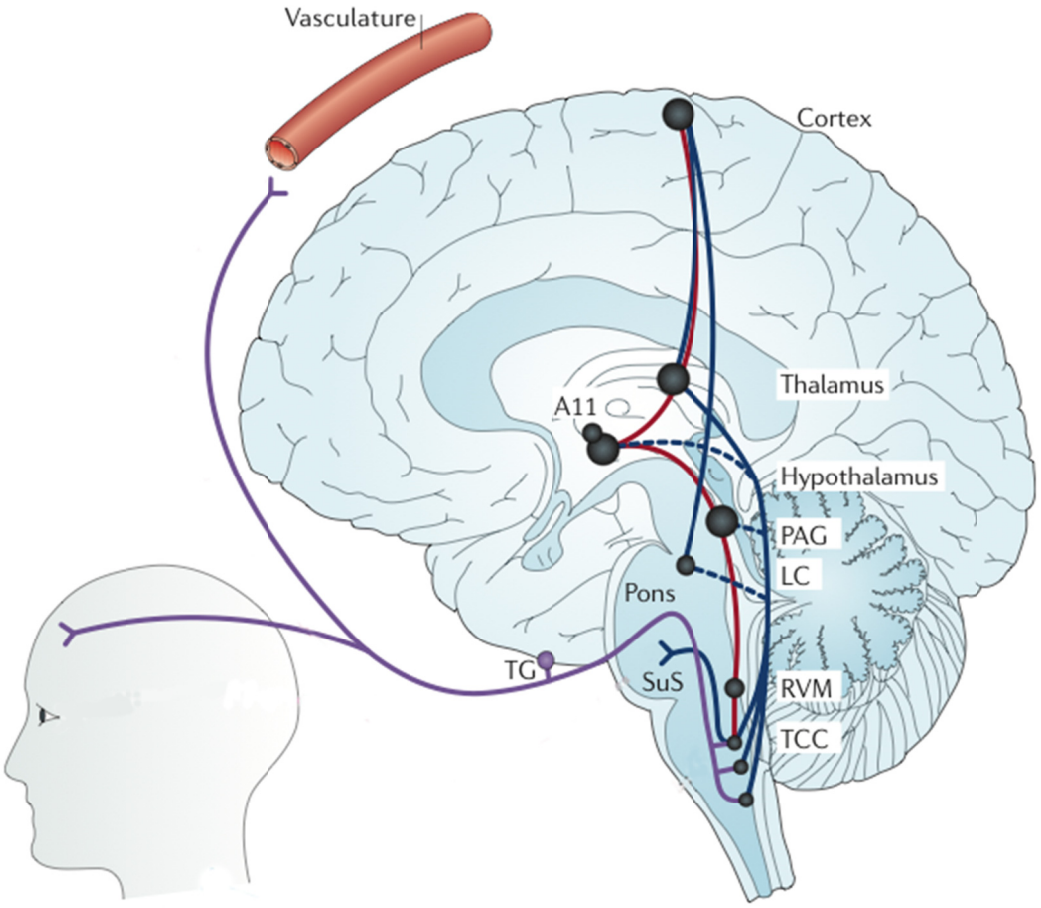

Figure 1. Structures and neuronal pathways involved in the pathophysiology of migraine.

Sensory afferents from the head (dural-vascular structures) (violet line) travel through the trigeminal ganglion (TG) and synapse on second-order neurons in the trigeminocervical complex (TCC). The TCC extends from trigeminal nucleus caudalis to the caudal portion of the dorsal horn of the C2 spinal cord. TCC neurons project the thalamus and from here to the cortex for the subjective perception of pain (blue line). Second-order neurons in the TCC also send direct projections to various structures in the brainstem, including the locus coeruleus (LC) and periaqueductal grey (PAG). Sensory modulation can occur by descending pathways (red line) such as those from hypothalamus, PAG, LC and nucleus raphe magnus (RVM). Modified from Akermann *et al.*, 2011.

Trigeminal activation

It is widely accepted that migraine headache occurs through the activation of the trigeminal sensory afferents that innervate the cranial tissues, especially the meninges (Fig.1). These nerve fibers also innervate meningeal blood vessels and they are called trigeminovascular afferents. Potassium and hydrogen ions released during abnormal cortical activation (e.g. cortical spreading depression: CSD) in proximity of the sensory endings innervating the dura

may lead to the activation of the meningeal nociceptors, either through direct depolarization or through activation of nociceptive receptors (e.g. vanilloid receptors or acid-sensitive ion channel receptor) (Lauritzen, 1994). Activation of the peripheral endings of trigeminovascular afferents leads to the subsequent activation of second-order neurons in the TNC and in the two uppermost segments of the cervical spinal cord. Sensory information is then carried rostrally to higher brain structures that mediate the perception of pain. The activation of the TGVS also results in the release of vasoactive peptides from the peripheral nerve endings. Calcitonin gene-related peptide (CGRP) and substance P (SP) both play a critical role for the induction of neurogenic inflammation. Neurogenic inflammation describes a sterile, inflammatory event that occurs as a result of sensory nerve activation and neuropeptide release from these fibers. It is characterized by vasodilation, plasma protein extravasation, and release of proinflammatory and inflammatory molecules from resident mast cells (Raddant *et al.*, 2011).

Peripheral and central sensitization

Throbbing pain, a cardinal feature of migraine, might be due to an increased sensitivity (sensitization) of trigeminovascular afferents to mechanical stimuli. Indeed it has been shown that these afferents can become mechanically hypersensitive after exposure of their dural receptive field to inflammatory agents (Strassman *et al.*, 1996). A large number of chemical mediators can promote the excitation and sensitization of nociceptors. Mediators such as bradykinin, histamine, serotonin (5- HT), and prostaglandin E2 (PGE_2) have been shown to sensitize meningeal nociceptors to mechanical stimuli (Levy *et al.*, 2002). PGE_2 and 5-HT are thought to do this by modulating tetrodotoxin resistant (TTX-R) sodium currents through activation of the cAMP-PKA second messenger cascade (Gold *et al.*, 1998).
Studies in humans and in animals indicate that nociceptive sensitization during migraine attacks is not restricted to the periphery but occurs also at central sites specifically the terminal sites of primary trigeminal nociceptors in the TNC. In rodents, topical application of inflammatory agents onto the exposed rat dura, induces long-lasting sensitization of medullary dorsal horn neurons that receive convergent intracranial input from the dura and extracranial input from the periorbital skin. This sensitization results in intracranial as well as

extracranial sensory hypersensitivity, rendering an innocuous stimulus, such as brushing, very painful (Yamamura *et al.*, 1999). In many patients, migraine attacks are associated not only with throbbing pain but also with cutaneous allodynia, which describes pain evoked by normally innocuous stimuli (Burstein *et al.*, 2000). Allodynia is generally believed to originate from central sensitization (Woolf, 2011).

Interestingly, migraine patients with and without allodynia respond differently to acute migraine treatments (Burstein *et al.*, 2011). Patients who do not report allodynia during migraine are highly responsive to triptans. When administered immediately after the onset of the migraine attack, they can render the patient pain-free within 2 hours of treatment. Patients whose migraine headache is accompanied by cutaneous allodynia become increasingly resistant to triptan therapy during the course of the attack. They fail to respond to triptans if treatment is delayed until they have fully developed allodynia over a period of several hours (Burstein *et al.*, 2004a). In contrast to triptans, infusion of COX1/COX2 inhibitors was shown to block sensitization in meningeal nociceptors and suppressed ongoing sensitization in spinal trigeminovascular neurons in the rat. (Jakubowski *et al.*, 2007). Moreover the infusion of the COX1/COX2 inhibitor ketorolac has been shown to abort both the headache and the allodynia suggesting that parenteral NSAID administration acts in the dorsal horn to inhibit the central neurons directly and reduce the synaptic input from the peripheral trigeminovascular neuron.

What activates the trigeminovascular system?

While there is a general consensus on the critical involvement of the trigeminovascular system, the causes of this activation are less clear. Two competing concepts favor a primary dysfunction either in the cortex or the brainstem.

CSD and Aura

As described above, in about one third of migraine patients, headache attacks are preceded by a so called aura, a transient cortical dysfunction which is closely linked to a phenomenon called cortical spreading depression (CSD). This event may be responsible for the activation of the trigeminovascuar system (Lauritzen, 1994). CSD is characterized by a wave of transient but strong neuronal activation propagating at a slow velocity of 2-6 mm min^{-1} across the cortex, followed by a suppression neuronal activity, lasting for minutes (Fig.2). CSD was first described 40 years ago by the Brazilian scientist Aristides Leao, who induced and recorded CSD in anaesthetized rabbits (Leao, 1947). Leao measured variations in the voltage and in the spontaneous local electrical (EEG) activity of the cortex after a period of repetitive electrical stimulation, describing the peculiar progression of the depression. In rodents, CSD is readily detected as a depolarizing shift in the direct current potential measured through electrodes inserted into the cortex. CSD triggers employed in animal studies include mechanical alteration of the cortex, exposure to high KCl solutions and electrical stimulation (Tfelt-Hansen, 2010). Unlike in animals, changes in direct current (DC) potential have been difficult to detect in humans as the typical detection techniques require invasive electrode measurements. However, using functional magnetic resonance (MRI), CSD-typical cerebrovascular changes could be recorded in the cortex of migraine patients while experiencing a visual aura. Blood oxygenation level-dependent (BOLD) signal changes during aura resemble different features of CSD. A strong temporal correlation was detected between the onset of the aura (scintillations starting in the paracentral visual field) and the initial increase in the BOLD signal reflecting vasodilation and cortical hyperemia. The following decrease in the local BOLD signal, which possibly reflects a vasoconstriction, correlated temporally with the scotoma that followed the scintillations (Hadjikhani *et al.*, 2001). The temporal and spatial changes in occipital cortex BOLD signals thus nicely resemble the visual disturbances propagating across the visual field. In addition,

magnetoencephalography (MEG) has also been successfully utilized to demonstrate that the occurrence of CSD correlates with visual aura in migraine patients. Focal DC potential shifts propagating across the cortex during a spontaneous visual aura were shown to cause slow changes in the cortical magnetic field (Bowyer *et al.*, 2001). The MEG field changes were comparable to those previously detected during CSD in animal models (Bowyer *et al.*, 1999). These observations strongly suggest that visual aura arises from CSD events in human visual cortex. If we consider CSD as the initial event causing migraine headache, then the question arises: what is the primary dysfunction in migraine without aura? It is possible that CSD, or CSD like events, generated within clinically silent brain regions may activate the trigeminal system as in the case of migraine with aura (Vecchia *et al.*, 2012). Evidence in favor of this hypothesis is based on studies that show cortical perfusion changes during migraine without aura attacks similar to those observed during migraine aura. In this study, positron emission tomography (PET) revealed unilateral hypoperfusion in occipital areas with a tendency to spread rostrally from the occipital cortex to parietal regions at a constant rate (Woods *et al.*, 1994). Moreover a spreading hyperemia was observed in a study using BOLD functional MRI during five attacks of MO triggered by visual stimuli (Cao *et al.*, 2002). More recently, using the PET technique, a relative hypoperfusion was detected in the occipital cortex of migraine patients during MO attacks (Denuelle *et al.*, 2008). In light of their results, the authors propose that the primary event in both MO and MA may arise from hypoperfusion triggered by the activation of brainstem nuclei. In MA, cortical susceptibility to hypoperfusion could then cause CSD producing the visual symptoms seen in MA.

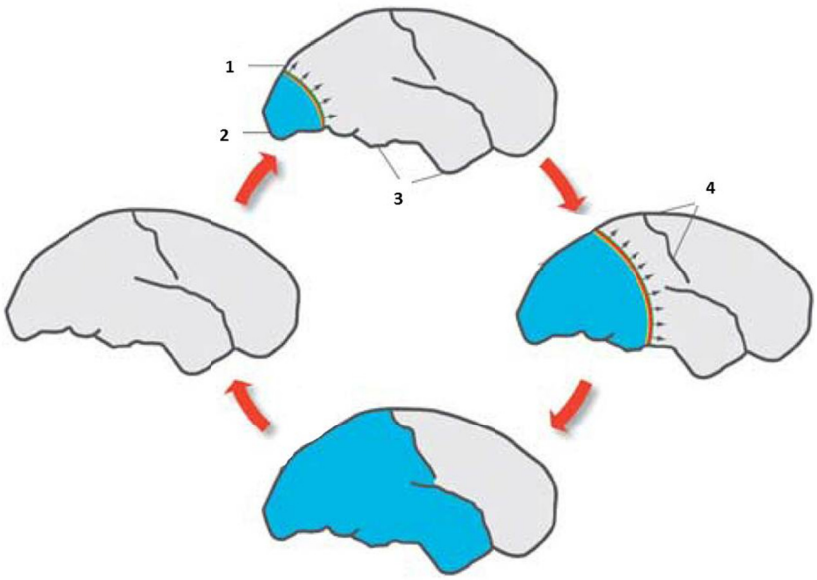

Figure 2. CSD propagation in migraineurs.

Lateral views of the human brain at different intervals after the beginning of the attack. Light blue area represents area of decreased regional cerebral blood flow, corresponding to the depression of cortical activity. The direction of propagation of CSD is indicated with arrows. At the start of migraine attack, a CSD emerges in the occipital pole in patients with a visual aura and spreads anteriorly (1,2). Cerebral blood flow in regions not invaded by CSD remains normal (3). The region of reduced CBF expands and stops on reaching the central sulcus (4). (Modified from Lauritzen et al., 2011)

Brainstem dysfunctions

An alternative hypothesis proposes that the primary cause of migraine headache lies in an episodic dysfunction of brainstem nuclei that are involved in the central processing and control of trigeminal nociception. Nociceptive signals from the dura mater are conveyed by trigeminal nociceptors to the TNC, from where they travel through ascending projections to pain processing centers in the rostral ventromedial medulla (RVM), periaqueductal gray (PAG) and thalamus. These nociceptive signals are under descending control by 'on' and 'off' cell projections from the ventrolateral PAG, via the RVM (Pietrobon *et al.*, 2003). For some forms of chronic non-headache pain, it has been shown that activation of descending brainstem pathways facilitates pain transmission. In particular, the descending modulation of spinal responses by the brainstem is likely to exert both facilitatory and inhibitory influences on spinal nociceptive processing (Millan, 2002). A number of studies have demonstrated activation of various brainstem structures (via an increase in regional cerebral blood flow, rCBF) during migraine attacks. For example, Weiller and colleagues reported a

significant rCBF increase in the dorsal raphe nucleus, PAG and locus coeruleus (LC) in migraine patients (Weiller *et al.*, 1995). The red nucleus and the substantia nigra were also foci of increased rCBF in MO and MA (Cao *et al.*, 2002). Considering these reports, it is plausible that the brainstem may contribute to both inhibitory and facilitatory modulation of migraine pain (Akerman *et al.*, 2011). Thus, dysfunctions of brainstem nuclei that are involved in the central control of pain might exert a permissive role by favoring central trigeminal hyperexcitability.

Cortical excitability in migraine patients

A number of clinical studies and observations argue in favor of the cortex being the primary origin of migraine attacks. In many migraine patients, cortical dysfunction occurs not only during aura but mild impairments can also be detected during the interical phases. Such interictal hyperexcitability has been found in the occipital cortex using transcranial magnetic stimulation (TMS) in both MA and MO patients. These studies showed that MA and MO patients were more prone to the development of phosphenes (typical luminous perception) after magnetic stimulation than healthy control patients (Giffin *et al.*, 2002). Impaired habituation has been consistently reported in migraine, extending to all sensory modalities, and is considered to represent a neurophysiological hallmark of the disease (Schoenen *et al.*, 2003). Habituation is defined as a decline in the response to repetitive stimuli. Visual activation monitored by magnetoencephalography and functional MRI-BOLD has confirmed abnormal excitability of widespread brain regions including the occipital, occipitotemporal, and occipitoparietal cortex, with consequent triggering of the neuroelectric accompaniments of aura symptoms (Welch, 2003). In addition, a shortened silent period in facial muscles was observed in migraine patients compared to healthy subjects. Since facial-muscle silent period is considered to be a purely cortical phenomenon, it has been proposed that its reduction in migraine could represent an abnormal excitability of the motor cortex (Curra *et al.*, 2007). It has been hypothesized that increased cortical excitability in migraine patients might result from reduced inhibition of cortical circuits (Aurora *et al.*, 2007; Coppola *et al.*, 2007). Furthermore this hyperexcitability may not be restricted to the cortex but might also extend to sub-cortical structures (Brighina *et al.*, 2009)

Human genetics of migraine

Important insight into the question of why some individuals (brains) are more susceptible to CSD and migraine than others has come from human genetic studies on migraine. Heritability of migraine susceptibility has been demonstrated already in population-based family surveys and in twin studies (Stewart *et al.*, 1997; Ulrich *et al.*, 1999). In the majority of patients, migraine is a polygenic multifactorial disorder. However, in certain rare cases a severe form of migraine, called familial hemiplegic migraine (FMH) has been described. FHM is an autosomal dominant trait with 80-90% penetrance (Ducros *et al.*, 1995; Riant *et al.*, 2005). Patients suffering from FHM experience similar migraine attacks to patients with MA and additionally suffer from hemiparesis during aura (Wessman *et al.*, 2004). FHM attacks are characterized by motor aura symptoms typically consisting of unilateral motor weakness or paralysis that may last from minutes to weeks. FHM patients exhibit profound clinical variability in the severity and frequency of attacks even among individual of the same family. Emotional stress and minor head trauma are among the most common triggers of FHM attacks.

Underlying mutations have been associated with three genes (*CACNA1A* in type 1 FHM (FHM1), *ATP1A1* in FHM2 and *SCNA1A* in FHM3) (van den Maagdenberg *et al.*, 2004). The *CACNA1A* gene encodes for the α1 (pore forming) subunit of P/Q type Ca^{2+} channels. These voltage gated Ca^{2+} channel trigger the release of neurotransmitters at peripheral and central synapses. More than 50 mutations in the *CACNA1A* gene have been described in FHM1 patients. The best characterized mutation is the R192Q amino acid substitution which accounts for 75% of all FHM1 cases. In mice homozygous for this mutation, synaptic glutamate release and susceptibility to CSD are increased (van den Maagdenberg *et al.*, 2004). The Na^+/K^+ ATPase transporter encoded by the *ATP1A1* gene is expressed by both neurons and glia. All known FHM2 mutations result in a “loss of function” or a kinetically altered Na^+/K^+ pump, which leads to reduced uptake of ions and neurotransmitters from the synaptic cleft. This effect could contribute to increased neuronal excitability and CSD susceptibility (De Fusco *et al.*, 2003). In FHM3 patients, mutations in the *SCNA1A* gene, which encodes for the voltage gated sodium channel $Na_{v1.1}$, affect the channel kinetics,

causing a more rapid recovery from fast inactivation after depolarization resulting in increased frequency of action potentials firing, enhanced neurotransmitter release and increased cortical excitability (Dichgans *et al.*, 2005). These findings suggest that heightened cortical excitability may underlie increased migraine susceptibility in all three forms of FHM and possibly also in other more prevalent forms of migraine (de Vries *et al.*, 2009). However, convincing evidence that the *CACNA1A*, *ATP1A2* and *SCN1A* genes are involved in more common forms of migraine is still lacking.

The identification of mutations or polymorphisms linked to more common forms of migraine will hopefully improve our understanding of the molecular pathways involved in the pathology of migraine. Many candidate genes have been proposed so far. For instance, *NOTCH3* is the causative gene for CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalophathy). The prevalence of migraine in CADASIL is slightly higher than in the general population. It has been proposed that the *NOTCH3* mutations act as a migraine aura susceptibility gene by itself (Liem *et al.*, 2010). Another gene of interest is *KCNK18* which encodes for the TRESK two-pore potassium channel protein. These channels are involved in the generation of potassium leak currents which act to control the resting membrane potential (Enyedi *et al.*, 2010). A mutation in TRESK channel was found to underlie migraine with aura in a large family. All the affected members carried the mutation whereas 8 relatives, who did not have migraine, possessed a normal TRESK gene (Lafreniere *et al.*, 2010). According to recent findings, TRESK channels could play a role in controlling the excitability of TG neurons during inflammation, thus contributing to migraine pathology (Enyedi *et al.*, 2012).

Experimental models of migraine

A major challenge in mechanistic migraine research and in preclinical drug development studies on migraine is the identification of an appropriate animal model of the disease. Numerous attempts have been undertaken to develop such animal models (Olesen *et al.*, 2012b). Neurovascular models assume that the pain during migraine is caused by the activation of trigeminal afferents innervating the meninges. Electrical, chemical and inflammatory stimulation have been used at the level of the dura to mimic nociceptor

activation and sensitization during headache (Troltzsch *et al.*, 2007). Electrophysiological recordings in the TNC have shown that topical meningeal application of an inflammatory soup composed of different pro-inflammatory substances (such as prostaglandin E2 and bradykinin) was able to activate the trigeminal system. This model has been validated through pharmacological modulation by sumatriptan, which proved efficacious in blocking the enhanced sensitivity to mechanical stimuli caused by the inflammatory soup-mediated trigeminal activation (Burstein *et al.*, 2004b).

Another recently developed animal model of migraine used the intravenous injection of glyceryl trinitrate (GTN) in awake freely moving rats (Ramachandran *et al.*, 2012). GTN infusion reliably evokes migraine attacks in humans. Nitric oxide (NO) liberated from GTN is considered to be responsible for the underlying mechanism in this model (Thomsen *et al.*, 2001). In rats, the infusion of GTN induces an up-regulation of c-*fos* (a marker of neuronal activation) mRNA and protein levels in the TNC. The changes in c-*fos* expression could be reversed by the pre-emptive treatment with the anti-migraine drug sumatriptan. The problem with models such as the two above is that it is unknown to what extent the pharmacological stimulation resembles the natural course and cause of the disease.

Genetically engineered mice carrying mutations that predispose them to migraine attacks are another option and may circumvent these problems. The advance of genetic insight into migraine pathophysiology and improvements in experimental read-outs have greatly contributed to the development of new genetically modified mice. For example, mice have been generated which carry a R192Q point mutation targeted to their *cacna1a* gene (*cacna1a* (R192Q) knock-in mice). These mice constitute the first and most widely used genetic mouse model of migraine. In homozygous point-mutated mice this mutation confers a gain-of-function on P/Q-type Ca^{2+} channel currents, increases glutamatergic neurotransmission and increases susceptibility to electrically evoked CSD (van den Maagdenberg *et al.*, 2004).

Another mutation described in FHM1 patients has been introduced into the mouse genome to create S218L knock-in mutant mice (van den Maagdenberg *et al.*, 2010). These mice show an even lower threshold for CSD compared to R192Q mice and exhibit severe hemiplegia after the CSD induction, partly reflecting the characteristics observed in the clinical

phenotype. Patients with the S218L mutation suffer from severe neurological symptoms, including ataxia, susceptibility to seizures, delayed brain edema, and fatal coma after minor head trauma (Kaja *et al.*, 2010). Recently the first FHM2 knock-in mouse model carrying the human W887R mutation in the *Atp1a2* gene has been generated (Leo *et al.*, 2011). Mice heterozygous for the mutation displayed an increased CSD susceptibility and velocity. The phenotypes observed in the three genetic mouse models of migraine described here suggest that increased levels of glutamate and K^+ in the synaptic cleft mediate an increased propensity for CSD. The increased susceptibility for CSD could well explain the aura experienced by FHM patients. It remains to be established whether this would also result in a more readily activated TGVS and thereby the headache. A significant and so far only partially met need in migraine research concerns the availability of read outs allowing for the assessment of migraine pain in animals. Indeed very few reports exist on measurable behavioral correlates of migraine. The majority of studies on migraine pain and trigeminovascular sensitization employ a classical immunohistochemical approach to study trigeminal activation and sensitization. As mentioned above, immunoreactivity against c-*fos* has been extensively used as a marker neuronal activation in pain experiments (Presley *et al.*, 1990). C-*fos* expression within the trigeminal nucleus can be induced by mechanical, chemical or electrical stimuli applied to the dura (Moskowitz *et al.*, 1993). Analysis of c-*fos* activation can therefore be considered as a validated read out for the activation of the TGVS. Recently, Mogil and colleagues observed a baseline pain face in restrained *cacna1a* (R192Q) mutant mice compared to wild-type mice suggestive of endogenous pain in the mutant mice (Langford *et al.*, 2010; Sotocinal *et al.*, 2011). *Cacna1a* (R192Q) knock-in mice have also been reported to display aversion to light (i.e. photophobia) in a plus maze test and an increased head grooming behavior when compared to wild-type controls (Chanda et al., 2008).

GABAergic and glycinergic transmission in central trigeminal sensitization

A loss of inhibitory synaptic control has been shown to contribute to central pain sensitization associated with a variety of different pain states (Zeilhofer *et al.*, 2012). In the mammalian central nervous system inhibitory neuronal control is achieved through the two

fast inhibitory neurotransmitters GABA and glycine. Both molecules act at inhibitory synapses inducing hyperpolarization and a shunting conductance at the postsynaptic level. With the use of pharmacological tools, it has been possible to demonstrate that the blockade of GABAergic and glycinergic inhibition induces signs of severe pain hypersensitivity. For example, intrathecal injection of strychnin, an antagonist of glycine receptors, has been shown to cause heat hyperalgesia in the rat. A similar pain phenotype has been reported in rats after the intrathecal application of either bicuculline or picrotoxin (Beyer *et al.*, 1985; Roberts *et al.*, 1986).

As mentioned above, facial allodynia occurs frequently during migraine attacks (Burstein *et al.*, 2000). Allodynia is pain evoked by activation of non-nociceptive fibers that do not directly innervate central nociceptive neurons. It is of central origin and has often been suggested to be caused by reduced inhibitory control in the spinal dorsal horn or the trigeminal nucleus. In fact, strong allodynic responses can be triggered by pharmacological blockade of glycinergic inhibition in the TNC (Miraucourt *et al.*, 2007). Nociceptive afferents coming from the meninges and tactile afferents from the facial skin terminate in different laminae of the TNC. The pathways relaying the signals from both areas are normally propagated through distinct ascending pathways. This separation depends on intact functional inhibition in the TNC. In the analogous structure of the spinal cord, the spinal dorsal horn, it has been demonstrated that processes secondary to peripheral inflammation can compromise this inhibitory control. Work in spinal cord slices demonstrated that glycinergic neurotransmission is reduced in mice with peripheral inflammation (Muller *et al.*, 2003). In inflammatory conditions the induction of COX2 in the spinal cord leads to an increase in the production of PGE_2, which in turn activates postsynaptic prostaglandin receptors of the EP2 subtype and finally causes the inhibition of α3-containing glycine receptors through PKA-dependent phosphorylation (Harvey *et al.*, 2004) (Fig.3).

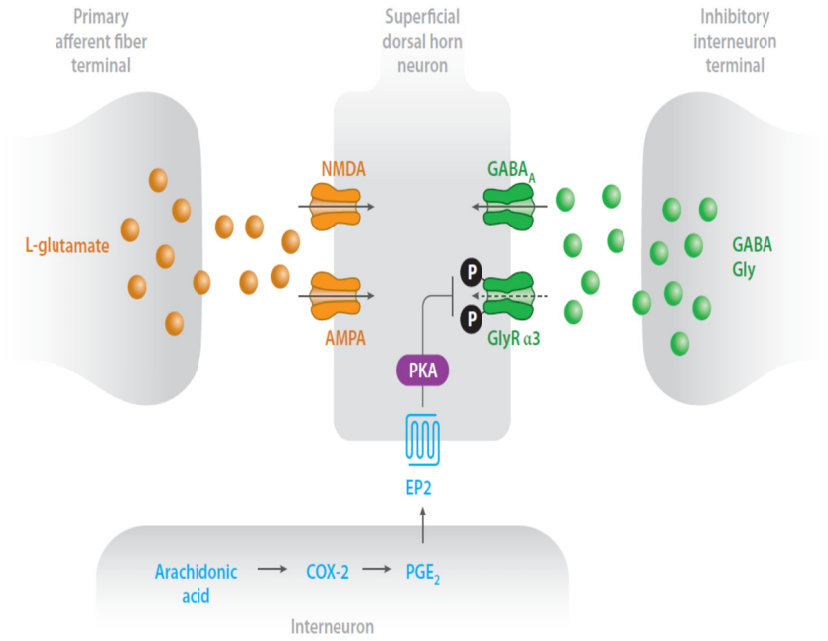

Figure 3. Mechanism of spinal cord disinhibition in inflammatory conditions.

Glycine and GABA released from presynaptic nerve terminals of GABAergic and glycinergic interneurons bind to heteromeric postsynaptic $GABA_A$ and GlyR receptors inducing channel opening and Cl^- influx. PGE_2 produced during the course of inflammation binds to EP2 receptors, expressed on intrinsic spinal cord neurons, which specifically couple to Gs proteins. Their signaling activates PKA through the increases of intracellular cAMP. PKA phosphorylates GlyRs containing α3 subunits, thus mediating their functional inhibition. This reduction of inhibitory postsynaptic response induces facilitation in the transmission of nociceptive input to the brain. (from Zeilhofer et al., 2012)

Mice lacking the EP2 subtype of PGE_2 receptors or the glycine receptor α3 subunit, two key elements of the underlying signal transduction pathway, recover significantly faster than wild-type mice from inflammatory hyperalgesia induced by subcutaneous zymosan A or complete Freund's adjuvant injection (Reinold *et al.*, 2005). The observed phenotypes are in agreement with the data obtained in PKA deficient mice and in normal mice following pharmacological blockade of PKA. Specifically, diminished inflammatory hyperalgesia was observed in mice lacking neuronal PKA (Malmberg *et al.*, 1997) and repeated intrathecal injection of a selective PKA inhibitor was able to reverse CFA-induced thermal hyperalgesia (Yajima *et al.*, 2003). In this context it is interesting to note that the allodynia associated with migraine attacks can be readily reversed by COX1/COX2 inhibitors, most likely by suppressing central sensitization in the TNC (Jakubowski *et al.*, 2005). Due to the importance of loss of inhibition in different pain states including migraine pain, a rational approach to address these forms of pain could therefore focus on the pharmacological

restoration of synaptic inhibition (Zeilhofer *et al.*, 2012). In addition to inhibitory glycinergic synapses, which are found mainly in the spinal cord and brain stem but for which no well-suited modulators are available, ionotropic $GABA_A$ receptors represent a promising target.

$GABA_A$ receptors

Ionotropic $GABA_A$ receptors are heteropentameric chloride-permeable ion channels composed from a repertoire of 19 subunits. Most $GABA_A$ receptors in the CNS are benzodiazepine-sensitive and contain two α subunits (α1, α2, α3 or α5), two β subunits and one γ2 subunit, of which the latter together with one of the α subunits forms the benzodiazepine binding site. A smaller subset of $GABA_A$ receptors that is mainly located extrasynaptically contains, instead of γ2, a δ subunit together with β3 and α4 or α6 (in the cerebellum). The generation of four $GABA_A$ receptor point-mutated mice carrying diazepam-insensitive $GABA_A$ receptor α subunits has greatly facilitated the attribution of the different *in vivo* actions of benzodiazepines to different $GABA_A$ receptor isoforms. These mice allowed attribution of the sedative actions of benzodiazepines to $GABA_A$ receptors containing α1 subunits, whereas the anxiolytic actions require the activation of $GABA_A$ receptors containing α2 subunits. By using these mice, it was also possible to attribute the antihyperalgesic effects of diazepam in the spinal cord to $GABA_A$ receptors containing α2 and α3 subunits. (adapted from Zeilhofer *et al.*, 2009)

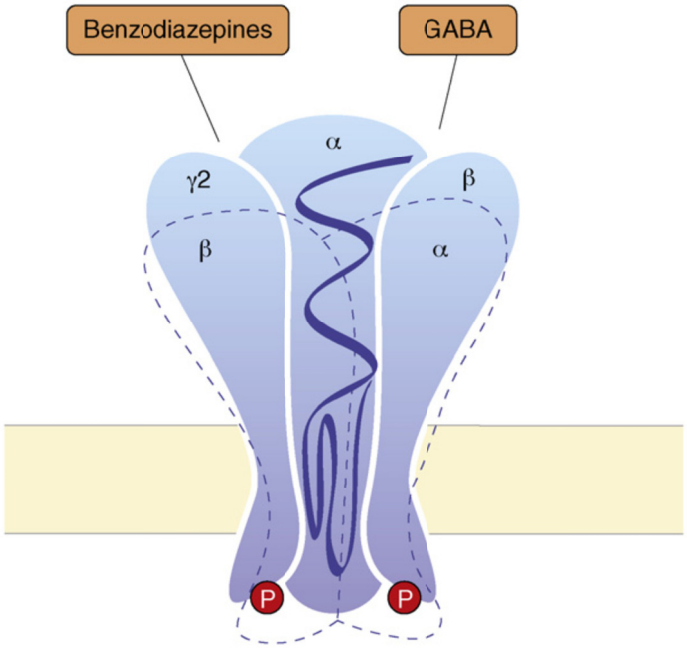

Figure 4. Molecular composition of benzodiazepine-sensitive $GABA_A$ receptors.

Indeed, drugs that enhance GABAergic transmission improve the outcome of both inflammatory and neuropathic pain (Zeilhofer *et al.*, 2009).

Since central sensitization in migraine may be explained by a loss of inhibition at the brainstem level, a similar pharmacological approach could be applied in migraine patients. In a parallel project, I have therefore evaluated the potential antinociceptive effects of a non-sedative $GABA_A$ receptor partial agonist.

EXPERIMENTAL SECTION

Aims

1st Project:

The first aim of this thesis was to determine whether the EP2/GlyRα3 receptor pathway, which has already been shown to contribute to spinal disinhibition and sensitization associated with peripheral inflammation, could contribute to central sensitization at the trigeminal level in migraine pain. To this end we have compared the phenotype of $EP2^{-/-}$ and $GlyR\alpha3^{-/-}$ mice with the well established genetic mouse model of migraine, *cacna1a* (R192Q) using behavioral, morphological and electrophysiological experimental approaches. From these experiments we expect a better understanding of the role of central sensitization in migraine.

2nd Project:

Cortical spreading depression (CSD) has been suggested to be the electrophysiological correlate of aura and may be the initial migraine trigger. Despite the recent insight into mechanisms of CSD and subsequent trigeminal activation, it is still unknown what predisposes brains of migraineurs to CSD. In rodents, evoked CSD is detected as a depression in the EEG signal. Accordingly, events of spontaneous CSD should also cause a change in the EEG patterns, resulting in a depression of brain activity. The second aim of this thesis was therefore to study the occurrence of spontaneous CSD and possible cortical dysfunctions associated with migraine in freely moving mice. To this end, we recorded EEGs of wild-type and *cacna1a* (R192Q) mice with the NeuroLogger®, a miniaturized data logger that allows the recording of EEG from 4 channels in freely moving mice.

3rd Project:

A loss of inhibition is thought to contribute to the development of allodynia and hyperalgesia in different forms of pain. A pharmacological restoration of this loss of inhibition could therefore represent a strategy to reverse established signs of neuropathic, inflammatory or pain of other origins, such as migraine. Using $GABA_A$ receptor point-mutated mice, our group recently demonstrated that α2 or α3 $GABA_A$ receptor subtypes are the most relevant ones for the spinal antihyperalgesic action of classical benzodiazepines, while the sedation promoting α1 subtype did not contribute. Accordingly, non-sedative (α1 sparing) benzodiazepine site agonists should possess substantial antihyperalgesic activity in the absence of sedation.

The final aim of this thesis was therefore the evaluation of potential antihyperalgesic effects of the non-sedative benzodiazepine site ligand HZ166 in well-established models of neuropathic and inflammatory pain.

1st Project:

Assessment of the contribution of the EP2/GlyRα3 pathway to central sensitization associated with migraine pain.

Manuscript in preparation.

Abstract

Migraine is a complex neurovascular disorder of poorly understood pathophysiology. While the primary dysfunction causing migraine attacks is still unknown, significant progress has been made in the case of migraine aura and trigeminal activation, two important events associated with migraine. The former is most likely caused by a phenomenon called cortical spreading depression (CSD), a wave of sustained neuronal depolarization slowly propagating across the cortex. The latter is a generally recognized process involved in the development of migraine headache. Evidence from clinical studies suggests that trigeminal activation involves prostaglandin-dependent signaling either at the level of the peripheral sensory fibers (peripheral sensitization) or second order neurons in the trigeminal nucleus (central sensitization). Prostaglandin receptors of the EP2 subtype and the α3 subtype of strychnine-sensitive glycine receptors (GlyRα3) have previously been shown to be involved in central inflammatory pain sensitization at the level of the spinal cord. Here, we have investigated a possible contribution of EP2 and GlyRα3 receptors to central sensitization at the level of the trigeminal system. To this end, we compared the phenotypes of mice lacking either EP2 or GlyRα3 receptors ($EP2^{-/-}$ and $GlyR\alpha3^{-/-}$ mice) to those of point mutated mice carrying a gain of function mutation (R192Q) in the *cacna1a* Ca^{2+} channel gene. This point mutation is a frequent cause of familial hemiplegic migraine in humans. We first investigated photophobia and found that under our experimental conditions neither *cacna1a* (R192Q) nor $GlyR\alpha3^{-/-}$ or $EP2^{-/-}$ mice displayed increased aversion to light compared to corresponding wild-type mice. We next studied trigeminal activation by analyzing expression of the immediate early gene c-*fos* in the spinal trigeminal nucleus caudalis (TNC) following KCl-induced CSD. We found that initial trepanation induced considerable levels of c-*fos* expression in the TNC. Additional c-*fos* expression was elicited by multiple CSDs. This additional expression was comparable in *cacna1a* (R192Q), $GlyR\alpha3^{-/-}$, and $EP2^{-/-}$ mice. Finally, we compared the thresholds of CSD, its spreading velocity, and its latency after KCl application to the cerebral cortex. We found that *cacna1a* (R192Q) mice were more susceptible to chemically-induced CSD compared to wild-type littermates, confirming previous findings obtained with electrically-induced CSD (van den Maagdenberg et al., 2004). An unexpected finding was that $EP2^{-/-}$ mice exhibited increased susceptibility to KCl-

induced CSD comparable to that seen in *cacna1a* (R192Q) mice. This phenotype was mimicked by local application of an EP2 receptor antagonist (TG6-10-1) to the cerebral cortex. We hypothesize that diminished prostaglandin production and subsequently reduced EP2 receptor activation increases the susceptibility to CSD and to migraine attacks. Chronically diminished EP2 receptor activation may thus be relevant for the pathology of medication overuse headache (MOH), which occurs frequently in migraine patients taking non-steroidal anti-inflammatory drugs (NSAIDs) for prolonged periods of time.

Introduction

Migraine is an episodic neurovascular disorder that affects about 20% of the European population with women being affected about twice as frequently as men (Stovner *et al.*, 2006). It is characterized by recurrent unilateral and throbbing severe headache and is often associated with autonomic symptoms such as nausea, phonophobia and photophobia. In about 30% of patients, migraine attacks are preceded by a prodromal phase characterized by neurological dysfunctions, the so called aura, which frequently involves visual symptoms (migraine with aura, MA). The remaining 70% of patients do not consciously experience aura symptoms (migraine without aura, MO). To date, the therapy for migraine attacks is symptomatic and currently recommended medications for migraine treatment or prevention are efficacious only in a small subpopulation of patients (Pietrobon *et al.*, 2003). To improve migraine pharmacotherapy, a better understanding of the underlying pathomechanisms is needed.

An event which occurs early in the development of a migraine attack and which precedes the headache, is cortical spreading depression (CSD). It describes a wave of transient intense spike activity often starting in the occipital cortex and then slowly propagating rostrally. This increased spike activity is followed by a suppression of neuronal activity that can last for minutes (Bowyer *et al.*, 2001). Convincing evidence has accumulated indicating that CSD is the neurophysiological cause of aura symptoms (Tfelt-Hansen, 2010). It is possible that CSD may not only occur in patients with MA, but also in the others. In these patients CSD may affect merely cortical areas whose dysfunctions do not lead to readily experienced deficits. CSD may therefore be a critical event not only for the aura symptoms but it may also be responsible for the source of the headache.

It is widely accepted that CSD causes an activation of the trigeminovascular system (TGVS) ((Zhang *et al.*, 2011), see also (Ingvardsen *et al.*, 1997)) through an accumulation in the extracellular space of potassium ions, protons and certain messengers, such as nitric oxide and prostaglandins (Moskowitz, 2008; Strassman *et al.*, 1996). Activation of the TGVS manifests (1) in an excitation of primary trigeminal nociceptors, which probably accounts at least in part for the pain experienced during migraine attacks (Sanchez-Del-Rio *et al.*, 2006), and (2) in a biphasic change in the tone of meningeal blood vessels. The latter consists of an

initial hyperemic response and blood vessel dilation lasting for 1-2 minutes, and a longer lasting hypoemia and vasoconstriction (45-60 min) (Hadjikhani *et al.*, 2001; Shimazawa *et al.*, 1996). Nociceptor activation and vascular changes potentially interact via two different mechanisms. Vasodilatation may activate stretch-sensitive nociceptor endings, while activation of peptidergic trigeminal nociceptors causes the release of vasoactive neuropeptides (calcitonin gene-related peptide [CGRP] and substance P [SP]) from their peripheral terminals, which in turn dilate meningeal blood vessels.

In addition to these peripheral events, sensitization also occurs in the central trigeminal system. Studies in rats have shown that sensitization of the trigeminal nucleus in the brainstem results in intracranial (dura) and extracranial (periorbital skin) sensory hypersensitivity (Burstein *et al.*, 1998; Yamamura *et al.*, 1999). Clinical studies suggest that this central sensitization also contributes to altered sensory processing and pain in migraine patients (Burstein *et al.*, 2000). A central component is also supported by the observation that migraine attacks are associated with cutaneous allodynia in many patients. Furthermore, it has been shown that the ongoing allodynia associated with the pain during migraine attack is readily reversed by COX1/COX2 inhibitors, most likely by suppressing central sensitization in the spinal trigeminal nucleus (Jakubowski *et al.*, 2005). A pathway that contributes critically to central sensitization associated with peripheral inflammation has previously been described at the level of the spinal cord dorsal horn (Harvey *et al.*, 2004; Zeilhofer *et al.*, 2012). Induction of COX2 in the spinal cord leads to an increase in the production of PGE_2, which activates postsynaptic EP2 receptors and finally causes the inhibition of GlyRα3 through PKA-dependent phosphorylation. Given the contribution of central sensitization to migraine headache and the efficacy of COX inhibitors in migraine attacks, we hypothesized that EP2 receptor activation may also contribute to migraine pain. In particular, EP2 receptor activation and subsequent inhibition of glycinergic (GlyRα3-mediated) neurotransmission may represent a specific mechanism for increased excitability of the trigeminal system. Interestingly, it has previously been reported that trigeminal disinhibition induces facial tactile allodynia. Specifically, pharmacological blockade of glycinergic transmission at the trigeminal level has been shown to alter innocuous input to the superficial dorsal horn rendering normally tactile sensations painful (Miraucourt *et al.*, 2007). Here, we have

investigated the relevance of EP2 and GlyRα3 receptors in the central sensitization associated with migraine pain. To this end, we have compared the phenotypes of mice lacking either EP2 or GlyRα3 receptors to the *cacna1a* (R192Q) knock-in mice in behavioral, immunohistochemical and electrophysiological experiments.

Methods

Animals. All experiments were performed on 8 – 12 week old female mice kept at a 12 / 12 h light/dark cycle with free access to food and water. Permission for the animal experiments was obtained from the Veterinäramt des Kantons Zürich (ref. no. 91/2008 and 160/2011). All efforts were made to minimize animal suffering. In all experimental conditions, the observer was blinded to the genotype of the mice.

All the mouse lines were bred in the animal facility at the Institute of Pharmacology and Toxicology: wild-type C57BL/6 mice, knock-in *cacna1a* (R192Q) (van den Maagdenberg, 2004), EP2 (*ptger2*) receptor-deficient mice ($EP2^{-/-}$, Hizaki, 1999) and mice lacking the α3 subunit of inhibitory glycine receptor (*glra3*, $GlyR\alpha3^{-/-}$, Harvey et al. 2004). Since homozygous $EP2^{-/-}$ female mice are infertile, *cacna1a* (R192Q)/$EP2^{-/-}$ double mutant mice were obtained by crossing mice homozygous for *cacna1a* (R192Q) and heterozygous for EP2 receptor ($EP2^{-/+}$). *Cacna1a* (R192Q)/$GlyR\alpha3^{-/-}$ double mutant mice were obtained by crossing mice heterozygous for both mutations. Since the two genes are on the same chromosome, we have created a trans-heterozygote strain where the mutations of interest are balanced by each other. We have isolated a low percentage of animals that throw the double-mutant chromosome as a result of a crossover event.

Photophobia/Intellicage. To assess photophobic behavior an automated behavioral analysis was performed using the Intellicage apparatus (Newbehaviour AG). This is a commercially available system that consists of a large home cage equipped with four operant conditioning units located in the corners. In our experimental settings, this Intellicage was modified by addition of two smaller cages, called social boxes, connected to the central home cage via angled tubes. One Intellicage can house 8-16 mice carrying commercially available microchips for identification (Datamars), allowing them to be localized and to determine the preferred compartment without handling for up to 14 days. In a first set of experiments, the

preferences of mice for the 2 social boxes were analyzed either in the absence of any light stimulus (both compartments were dark for 24 h, one of them containing food and water) or with normal 12/12 light/dark in the main cage. Subsequently, different light conditions were set in the two social boxes: one of them was permanently dark, whereas the other contained food and water as a stimulus and was lit for 24 hrs (50, 100 or 300 lux). Preferences for the two small compartments were determined by exposing two groups of mice (*cacna1a*(R192Q) and wild-type littermates to different light intensities in the light social box (from 50 to 300 lux) in order to find a suitable light condition to evaluate photophobia. After selecting the light intensity, different groups of mice were tested for potential photophobic behavior. Time spent in the two different compartments was calculated as a percentage of total time and compared between the different genotypes.

Susceptibility to electrically-induced cortical spreading depression. Female mice (8-12 weeks old) were anesthetized with isoflurane (4% for induction, 3% or 1.5% during craniotomy and CSD protocol, respectively), mounted on a stereotaxic apparatus and continuously monitored for an adequate level of anesthesia. In animals, CSD can be triggered by focal stimulation of the cerebral cortex with electrical, mechanical or chemical stimuli. To record CSD, two holes were drilled into the skull (stimulation site: AP -3.3, ML 2.6. recording site: AP -1, ML 1.4, relative to the bregma) leaving the dura intact. The steady potential at the primary somatosensory cortex was recorded with a 0.9% saline filled glass electrode placed 300 μm below the dura (tip resistance 1-2 MΩ). An Ag/AgCl reference electrode was placed under the skin on the back of the neck. After at least 5 min of baseline recording, 100 ms current pulses of increasing intensity (20, 60, 80, 100, 200, 400, 600 and 800 μA) were applied via a silver bipolar electrode (250 μm tip diameter, 0.6 mm intertip distance) placed on the dura at the stimulation site (occipital cortex). A stimulus isolator/constant current unit was used to generate the stimulation current (Iso-stim 01D). The potential was recorded for 5 min after the stimulation using an EXT-02F amplifier (Npi Electronic GmbH). 100 ms long pulses of increasing intensity were applied at 5 min interval until a CSD event was evoked. The charge delivered with the first stimulation which activated a CSD was taken as the CSD threshold. After 5-10 min of wash out, the stimulation protocol was repeated. The final CSD threshold was calculated as the average between the two trials.

Susceptibility to chemically-induced cortical spreading depression. Female mice (8-12 weeks old) were anesthetized and prepared for CSD induction as described in the previous section but three holes were drilled into the skull (stimulation site: AP -3.3, ML 2.6; recording site 1: AP -1, ML 1.4; recording site 2: AP +1, ML 1.4; all relative to the bregma). Solutions containing different KCl concentrations were applied to the stimulus site starting at 5 mM and increasing with 7.5 mM step applied at 3 min intervals. If no CSD was observed after three minutes, the site was rinsed with 150 mM NaCl and solutions with 7.5 mM KCl increase were subsequently applied. Total osmolarity was kept constant at 300 mOsm with NaCl. Upon detection of a CSD event, the induction site was rinsed thoroughly with 150 mM NaCl, a baseline recording of at least 5 min was carried out and the threshold protocol was repeated. The average of the two KCl concentrations applied which activated a CSD was taken as CSD threshold. At the end of the experiment, CSD propagation velocity was estimated by measuring the distance between the recording electrodes and dividing this value by the elapsed time between onset of CSD at the different electrodes. For each recording session, mice from at least two different genotypes were analyzed. The genotype was revealed at the end of the analysis of the recording.

Effect of the EP2 antagonist TG6-10-1 on KCl-induced CSD susceptibility

Topical application of TG6-10-1. Female wild-type mice (8-10 weeks old) underwent surgical preparation as described in section "Susceptibility to electrically-induced…". The chemically-induced CSD protocol was performed as described in section "Susceptibility to chemically-induced". Upon the first CSD event, TG6-10-1 was applied to the dura on a cotton ball for 20 min. The compound was dissolved in 10% DMSO, 40% ddH_2O, 50% PEG400 and 5 µl of a 100 µM solution (TG6-10-1 or vehicle) was administered. After rinsing the window with 150 mM NaCl, the threshold protocol was repeated and the difference between the KCl concentration that induced the second and the first CSD event was calculated. For each recording session, mice from the drug and the vehicle group were analyzed. The experimenter was blinded to the treatment until the end of the analysis.

Systemic application of TG6-10-1. Female wild-type mice (8-10 weeks old) were injected intraperitoneally with either TG6-10-1 (5 mg/kg, dissolved in 10% DMSO, 40% ddH_2O, 50% PEG400) or vehicle in a total volume of 10 ml / kg body weight. About 30 minutes after drug

administration, mice underwent surgical preparation for CSD recording as described above. After electrode insertion, the steady potential was recorded for at least 5 min. Cortical stimulation with solutions containing different KCl concentrations was started 1 hour after drug injection. Following detection of a CSD event, the induction site was rinsed and the protocol was repeated. The average of the two KCl concentration applied activating a CSD was taken as CSD threshold. For each recording session, mice from the drug and the vehicle group were analyzed.

c-*fos* expression in the trigeminal nucleus caudalis after the induction of CSD

Cortical spreading depression induction. Female mice (8-12 weeks old) were anesthetized and prepared for CSD induction as in section 2. To record CSD, two holes were drilled in the skull on the right hemisphere using defined coordinates relative to the bregma (Stimulation: AP -3.3, ML -2.6, Recording (parietal): AP -1.0, ML +1.4). A glass-electrode was placed in the recording hole and the recording was started. A baseline recording of at least five min was performed, before the chemical stimulation in the occipital cortex (stimulating window) was started by application of KCl. In this experimental setup a concentration of 3M KCl was applied. A cotton ball soaked with 10 µl of the solution was gently applied on the occipital window. The application was performed six times in one hour, every nine minutes. Before starting a new application, the stimulating window was cleaned with 0.9% NaCl. At the end of the stimulation the skin was closed and the mouse was permitted to recover from anesthesia. For each group of mice, control-experiments were carried out using 0.9% NaCl instead of KCl. At least 2 mice for each condition (one treated with KCl and a control treated with 0.9% NaCl) were analyzed in the same recording session.

Perfusion and tissue preparation. One hour after completing the protocol of CSD induction, a transcardiac perfusion was performed. Mice were anesthetized with an overdose of pentobarbital (Nembutal > 50mg/kg i.p.). After rapidly opening the thorax to expose heart and major vessels, a blunted cannula (Ø 1mm) was inserted through the left ventricle into the ascending aorta. The right atrium was opened using a sharp needle and the peristaltic pump was started. The perfusion was started with ~ 20 ml PBS to flush away the blood followed by ~ 80 ml of fixative (containing 4% paraformaldehyde (PFA) and picric acid). The cervical spinal cord was dissected together with the brainstem and the whole brain starting from the lumbar

spinal cord. The collected tissue was post-fixed in 4% PFA in a falcon tube overnight at 4 °C. To prevent ice crystal damage during freezing, the tissue was stored in 30% sucrose in PBS overnight at 4 °C.

Sectioning. A sliding microtome (Zeiss Hyrax KS 34) was used to obtain 50 µm slices. First, the tissue was divided in two parts using a razor blade (~ at the level of locus coeruleus) to allow a parasagittal position of the tissue on the freezing stage. Sections were collected in series in a multi-well plastic box filled with ice-cold PBS. For long-term storage at -20°C sections were transferred in antifreeze solution (150g glucose, 300 ml ethylene glycol, add 500 ml of 50mM sodium-phosphate buffer (pH 7.4) and 200 mg sodium azide).

Immunoperoxidase staining (ABC method). To reduce background signal, quenching of endogenous peroxidases was performed incubating sections in 0.35% peroxide (H_2O_2) in ddH_2O for 5 min. The sections were transferred into a glass-dish containing the primary antibody-solution (1:10 000 anti c-*fos*-rabbit pAb, Calbiochem, 2% normal goat serum (NGS) and 0:2% Triton X-100) and incubated overnight at 4°C with continuous gentle shaking (100 rpm). After washing, the sections were transferred into a solution containing a biotinylated secondary antibody (biotin-conjugated goat anti rabbit IgG (Jackson Immunoresearch) and 2% NGS in tris-triton), and incubated for 30 min at room temperature with gentle shaking. After washing in Tris-Triton, slices were transferred to a solution containing the avidin-peroxidase-complex (ABC) (1% solution A, 1% solution B; Vectastain Elite kit standard, Vector Laboratories), mixed 30 min prior to use and incubated for 30 min at RT with agitation. The enzymatic reaction with diaminobenzidine (DAB) (50 mg/ml dH_2O) was catalyzed with H_2O_2 as described previously (Sidler et al. 2010). After 10 min the reaction was stopped. Slices were washed and mounted on gelatinized glass slides. After an overnight drying, sections were dehydrated using increasing ethanol concentrations (70%, 70%, 96%, 96%, 100%, 100%, 100%) and cleaned in xylene. Finally they were coverslipped with Eukitt mounting medium (Sigma Aldrich).

In order to compare mice with different genotypes and to avoid staining-variability, sections from mice of all different genotypes were stained on the same day. For the expression of c-*fos* in TNC after stress induction, staining was also performed for all genotypes in the same day. An automated upright slide scanning microscope (Zeiss Mirax Midi Slide Scanner) was

used to acquire images. Pictures were visualized with Panoramic viewer and processed with ImageJ.

GlyRα3 staining. Brain, brainstem and cervical spinal cord were dissected from 8-12 week old mice and rapidly frozen on dry ice. The frozen was then mounted in OCT embedding medium, 12 µm cryostat sections were cut, mounted on gelatin-coated slides, air dried, and stored at -20°C. Sections were fixed by incubation in 4% PFA (w/v) in PBS (pH 7.4) for 2 min followed by 30 min incubation in blocking buffer (10% normal goat serum NGS, 1% bovin serum albumin (BSA), 0.5 % Triton-X100 in PBS). Primary antibody incubation was performed overnight at 4°C in 3% NGS, 1% BSA, 0.5 % Triton-X100 in PBS. For GlyR α3 subunit-specific staining a rabbit polyclonal anti-α3 serum directed against the GlyR α3 C-terminus (Harvey, 2004) at a dilution of 1:200 was used. Co-staining for gephyrin was performed using a monoclonal anti-gephyrin antibody (Synaptic systems) at a dilution of 1:1000. After washing three times in PBS, sections were incubated for 1 hour in a solution containing the secondary antibodies (Cy3-conjugated Goat anti Rabbit IgG and Alexa Fluor 488 Goat anti Mouse IgG). For GlyR α3 subunit-specific staining a newly developed rabbit polyclonal anti-α3 serum directed against the GlyR α3 C-terminus was utilized at a dilution of 1:200 in 3% NGS, 1% BSA, 0.5 % Triton-X100 in PBS). After successive washes, the slides were immersed in distilled water to remove salt. After at least 2 hours of air drying, the sections were covered with Dako fluorescence mounting medium and slides were stored at 4°C. For each staining, control-experiments (staining without primary antibody) were performed. Images were acquired with a Zeiss ApoTome microscope. Pictures were subsequently processed with ImageJ software.

Results

Expression of GlyRα3 in the spinal trigeminal nucleus caudalis (TNC)

We first analyzed the expression of GlyRα3 receptors in coronal sections of the cervical spinal cord. As previously shown (Harvey *et al.*, 2004), GlyRα3 was expressed in the superficial laminae of the mouse dorsal horn. In spinal cord sections from GlyRα3$^{-/-}$ mice, no GlyRα3 immunoreactive signals were detected (Fig. 1A). Expression of GlyRα3 was evident along the entire spinal cord up to the caudal brainstem from the staining obtained in sagittal sections (Fig.1C).

The expression of GlyRα3 was then investigated at the level of the trigeminal nucleus caudalis in coronal sections. GlyRα3 immunoreactivity showed restricted expression in the superficial laminae of the TNC. The specific signal for GlyRα3 displayed in wild-type mice was completely absent in GlyRα3$^{-/-}$ mice (Fig.1B). Colocalization with gephyrin was found throughout the superficial laminae of TNC, indicating that GlyRα3 are synaptic and clustered by gephyrin (Fig. 1D). Collectively these results showed that GlyRα3 is expressed in the TNC and suggest that GlyRα3-mediated inhibition may participate in the control of the excitability at the trigeminal level.

A similar investigation/approach with respect to the EP2 receptor was hampered by the lack of suitable antibodies for immunofluorescence experiments. Nevertheless previous reports have demonstrated the presence of EP2 mRNA in the TNC and in the trigeminal ganglion (Myren *et al.*, 2012)

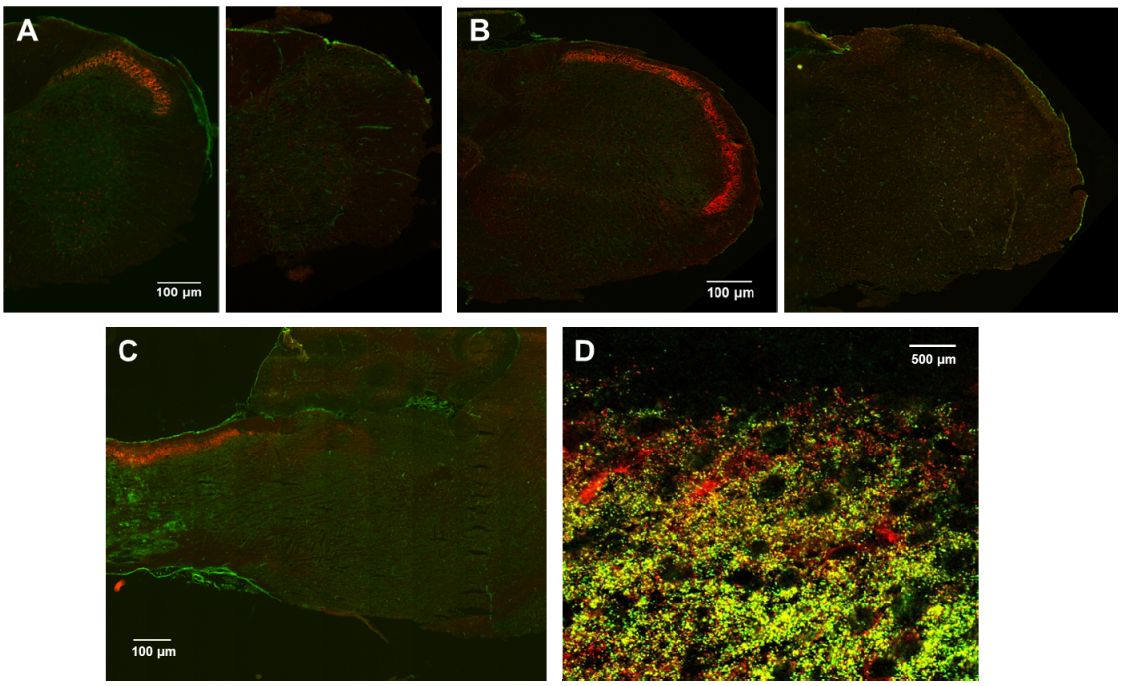

Fig.1 GlyRα3 localization in the spinal cord and brainstem. (A) Colocalization of GlyRα3 (red) and gephyrin (green) in the cervical spinal cord of a wild-type mouse (left). In the transverse section from a GlyRα3$^{-/-}$ mouse, GlyRα3 immunoreactivity is lost (right). (B) Colocalization of GlyRα3 (red) and gephyrin (green) in the superficial laminae of the spinal trigeminal nucleus caudalis of a wild-type mouse (left). The immunoreactivity for GlyRα3 is absent in the section from a GlyRα3$^{-/-}$ mouse (right). (C) Sagittal section of the rostral spinal cord, brainstem and brain stained for GlyRα3 (red) and gephyrin (green). (D) High-resolution image showing colocalization (yellow) of GlyRα3 (red) and gephyrin (green) in the TNC.

Analysis of photophobic behavior in the Intellicage

A major challenge in the course of this project was finding a suitable behavioral read-out for the different mouse models. Photophobia, defined as a hypersensitivity to light and usually including avoidance behaviors, is included as a criterion for migraine diagnosis in the International Headache Society classification (The International Classification of Headache Disorders, 2004). While the mechanisms underlying photophobia remain unknown, the trigeminal system appears to be involved (Kowacs *et al.*, 2001; Okamoto *et al.*, 2010). In a previous report, *cacna1a* (R192Q) mice have been shown to display photophobia in a modified elevated plus maze test (Chanda *et al.*, 2008). Here, we have used a new method that allows the investigation of potential light aversion in freely moving mice. We first analyzed the animal's preference for the two compartments in the absence of a light stimulus (both compartments were dark for 24 h, one contained food and water) in *cacna1a* (R192Q)

mice and wild-type littermates. We observed no differences in the preferences for the two different compartments between the two groups (data not shown). We then explored their behavior in the Intellicage with different light-intensities in the lit box (from 50 to 300 lux) to find a suitable condition for photophobia testing. (Fig.2A). The results did not reveal any significant differences between the various light intensities. We next investigated photophobic behavior in different groups of mice exposed to 100 lux intensity. *cacna1a* (R192Q), GlyRα3$^{-/-}$ and EP2$^{-/-}$ mice did not show any difference in the percentage of time spent in the light and in the dark box compared to wild-type mice. Thus, under our experimental conditions, *cacna1a* (R192Q) mice did not show photophobic behavior. Moreover GlyRα3$^{-/-}$ and EP2$^{-/-}$ mice did not display any aversion to light compared to wild-type or *cacna1a* (R192Q) mice.

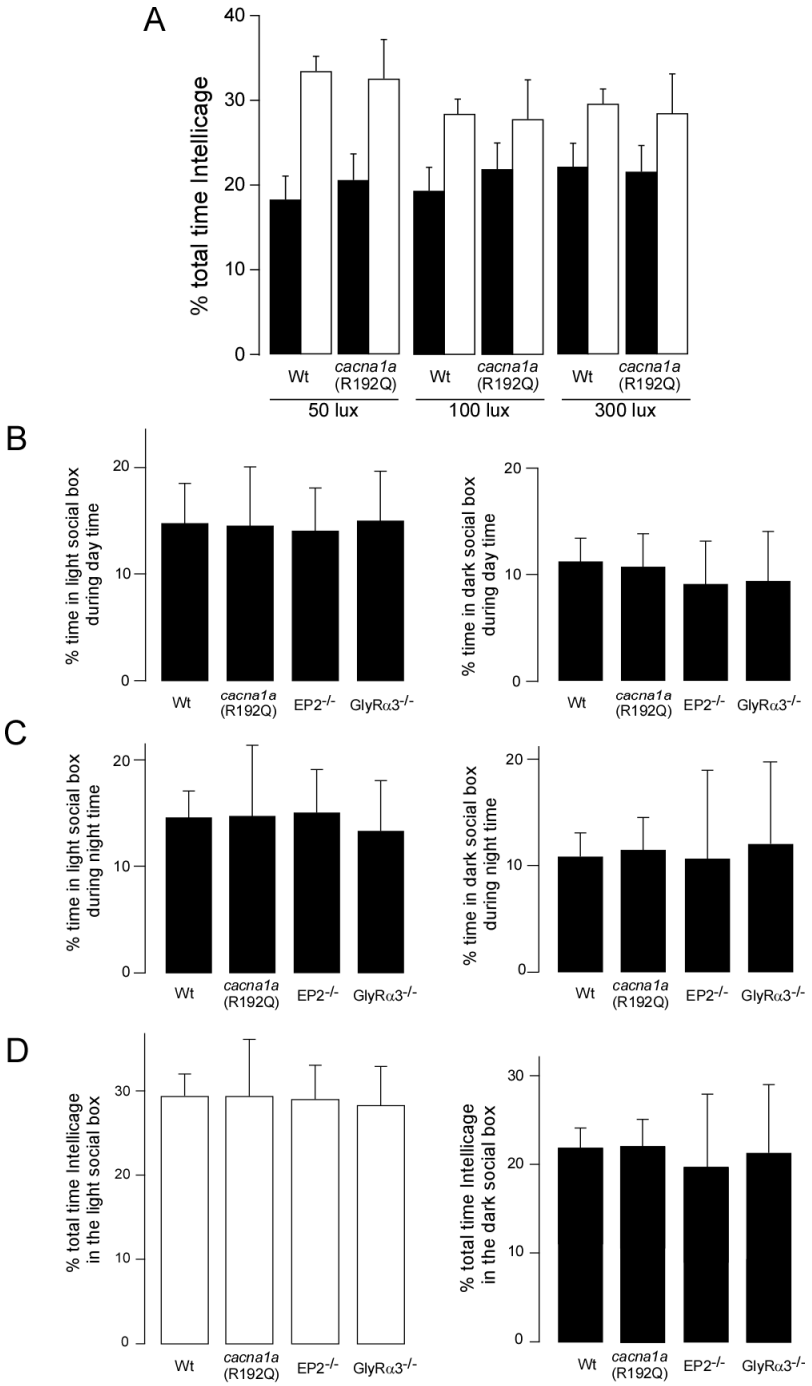

Fig.2 Assessment of photophobia in the Intellicage system. (A) Preferences for the dark (black) and the light (white) social box at different light intensities are depicted as percentages of total time spent in the Intellicage. Values are expressed as mean ± standard deviation. n=6 mice per group. (B) Percentage of total time in Intellicage spent in the light (left) and dark (right) social box during day time. Values are expressed as mean ± standard deviation (n=8 mice per group). (C) Percentage of total time in Intellicage spent in the light (left) and dark (right) social box during night time. Values are expressed as mean ± standard deviation (n=8 mice per group). (D) Percentage of total time in Intellicage spent in the light social box. Values are expressed as mean ± standard deviation (n=8 mice per group). G) Percentage of total time in Intellicage spent in the dark social box. Values are expressed as mean ± standard deviation (n=8 mice per group). Statistical comparisons were made using one-way ANOVA followed by Scheffe's post hoc test. No significant differences were found.

C-*fos* expression in the TNC after CSD or after stress-induction

Induction of c-*fos* expression is often used as an indirect measure of neuronal activity. c-*fos* expression has been used as a marker for the activation of the trigeminal system in various studies (Bolay *et al.*, 2002; Moskowitz *et al.*, 1993) and is therefore considered a marker of trigeminal nociception (Bergerot *et al.*, 2006). The effects of multiple CSD events on the expression of c-*fos* in the superficial laminae of the TNC were described previously in rats (Moskowitz *et al.*, 1993). We first counted the number of c-*fos* positive nuclei in naïve mice (without any surgical procedure or pharmacological treatment, n=4 mice per group, 6 sections per mouse). Very few cells were stained in all different genotypes (right plus left): 8.5 ± 4.5 in wild-type, 9.2 ± 3.5 in *cacna1a* (R192Q), 8.7 ± 4.1 in GlyRα3$^{-/-}$ and 8.1 ± 3.7 in EP2$^{-/-}$ mice (Fig.5A). We next analyzed the effect of CSD on the activation of the trigeminal system. To this end, we evaluated c-*fos* activation in the TNC after application of saline or multiple KCl-induced CSDs. Compared to naïve mice, all brainstem sections from both KCl and saline-treated mice displayed a strong bilateral c-*fos* expression.

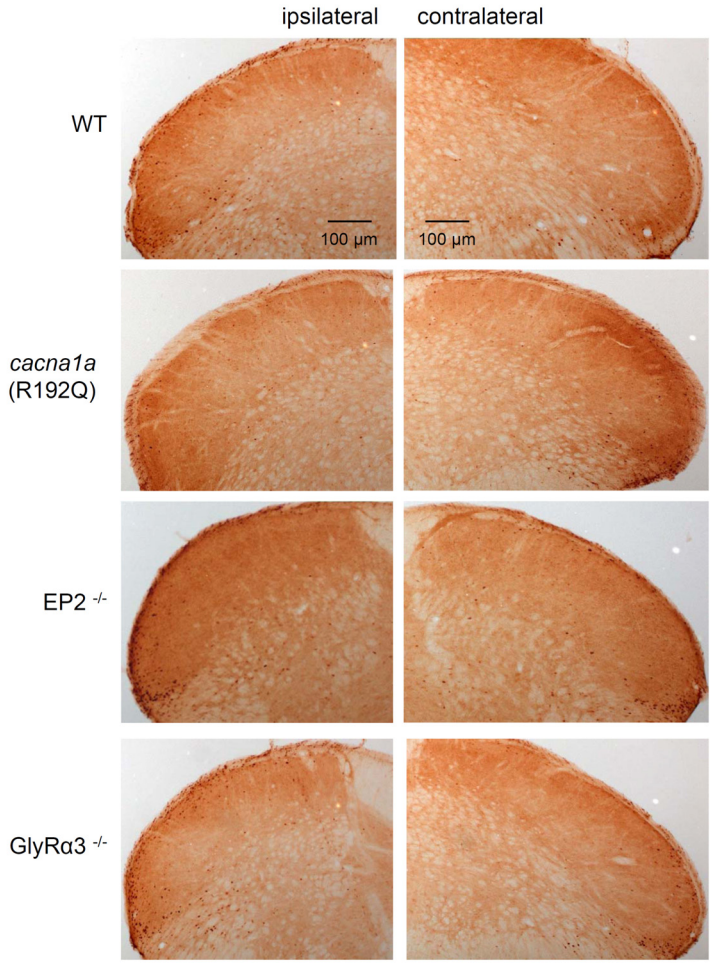

Fig.3 c-*fos* immunoreactivity in the TNC after surgery and saline application
Representative sections of the TNC from wild-type, *cacna1a* (R192Q), EP2$^{-/-}$, and GlyRα3$^{-/-}$ mice immunostained with an anti c-*fos* antibody (1:10000) after surgery and dural application of 0.09 % saline (control condition). Both ipsilateral (left) and contralateral (right) dorsal parts of the TNC are shown.

This was evident mostly in the superficial laminae I and II starting from the C2 and C1 level of the spinal cord and spreading to the entire TNC along the caudal-rostro axis. In all different genotypes, the craniotomy and the application of saline (control condition) caused a robust bilateral c-*fos* induction in the trigeminal nucleus: 51 ± 13.2 and 68 ± 13.9 (left and right, respectively) in wild-type, 46 ± 7.1 and 72 ± 9.7 in *cacna1a* (R192Q), 49 ± 11.6 and 66 ± 18.1 in EP2$^{-/-}$ and 80 ± 11.8 and 104 ± 9.9 in GlyRα3$^{-/-}$ mice (Fig.3 and Fig.5B).

After KCl application and the resulting occurrence of multiple CSD events, the number of c-*fos* positive cells in the TNC increased in all genotypes when compared to the respective control groups except for the GlyRα3$^{-/-}$ group. Numbers are as follows (left and right, respectively): 80

± 8.9 and 111 ± 11.6 in wild-type mice, 66 ± 6.4 and 98 ± 10.3 in *cacna1a* (R192Q), 83 ± 6.5 and 106 ± 8.5 in EP2$^{-/-}$, 72 ± 8.4 and 97 ± 10.7 in GlyRα3$^{-/-}$ mice (Fig.4 and Fig.5B). Interestingly, GlyRα3$^{-/-}$ mice showed a higher number of c-*fos* positive cells in the control groups compared to wild-type mice (51 ± 13.21 and 68 ± 13.9 (left and right, respectively) in wild-type and 80 ± 11.8 and 104 ± 9.9 in GlyRα3$^{-/-}$ mice. Moreover, CSD events failed to cause a further increase in the number of c-*fos* positive cells in the TNC of GlyRα3$^{-/-}$ mice.

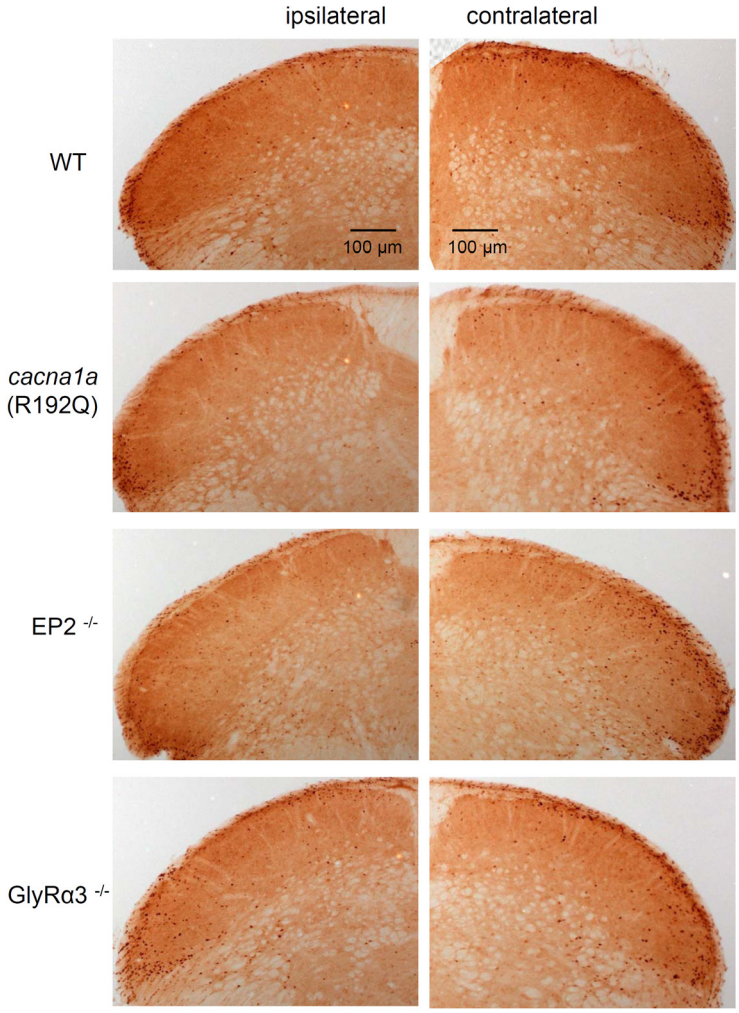

Fig.4 c-*fos* immunoreactivity in the TNC after multiple CSDs
Representative sections of the TNC from wild-type, *cacna1a* (R192Q, EP2$^{-/-}$, and GlyRα3$^{-/-}$ mice immunostained with an anti c-*fos* antibody (1:10000) after KCl application and consequent CSD induction. Both ipsilateral (left) and contralateral (right) dorsal parts of the TNC are shown.

These results showed that the surgical procedure had a direct effect on the c-*fos* expression in the trigeminal nucleus, suggesting that mechanical stimulation of the dura and/or endothelial vessels activates c-*fos* expression. Interestingly, the absence of GlyRα3 promoted

a further increase in the number of c-*fos* positive cells in control conditions, consistent with a role for GlyRα3-mediated synaptic inhibition in the control of the excitability in the TNC after injury (surgical procedure).

In order to more deeply investigate the role of GlyRα3 in the trigeminal activation, we have analyzed c-*fos* activation in a condition potentially causing CSD without any mechanical injury. Stress has been shown to be a trigger factor for migraine (Kelman, 2007) and c-*fos* expression in the brain after restraint stress in mice has been previously reported (Lauterborn, 2004). We explored the possibility that a potential migraine attack (and a relative CSD event) could be triggered by a stressor event. We therefore analyzed c-*fos* expression in the TNC of mice subjected to a 30 min restrain-stress protocol. Sections from wild-type, *cacna1a* (R192Q), GlyRα3$^{-/-}$ and *cacna1a* (R192Q) /GlyRα3$^{-/-}$ double mutant mice showed a very weak c-*fos* staining compared to the control condition after surgery. Moreover, only few positive cells were observed. c-*fos* positive cells were present throughout the whole TNC but notably they were not found in the superficial laminae. The total number of cells was on average between 10 and 20 for each site in the TNC (Fig.5C). Compared to wild-type (14.35 ± 2.75), *cacna1a* (R192Q) mutant mice show a trend towards a higher number of c-*fos* positive cells (18.87 ± 1.72, p= 0.35). In contrast, GlyRα3$^{-/-}$ mice showed a tendency towards lower number of c-*fos* positive cells (11.68 ± 1.13, p=0.53) compared to wild-type littermates. *cacna1a* (R192Q) / GlyRα3$^{-/-}$ double mutant mice displayed intermediate results (16.26 ± 1.97, p=0.38) (Fig.3C). Additionally, one GlyRα3$^{-/-}$ mouse and one *cacna1a* (R192Q) / GlyRα3$^{-/-}$ double mutant mouse did not show any positive cells.

These results demonstrated that the selected stress stimulus produced a weaker c-*fos* activation compared to surgery. Moreover the stress-induced c-*fos* activation was not significantly different from naïve mice without surgery. Thus, these data suggest that the mechanical injury provoked by the surgery represents a strong stimulus for c-*fos* activation which is possibly regulated by GlyRα3 mediated inhibition.

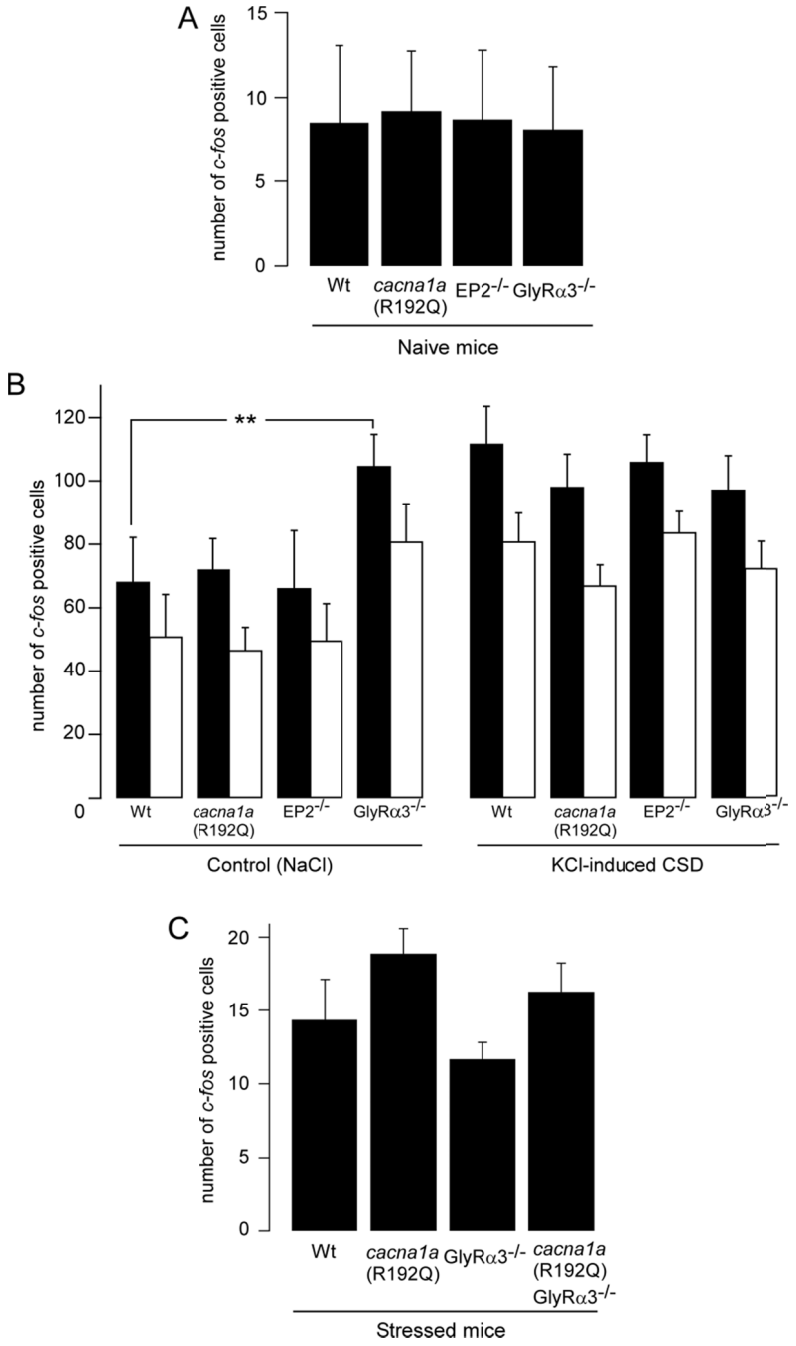

Fig.5 Quantitative analysis of c-*fos* expression in the TNC of naïve mice, under control conditions (surgery and saline application), after CSD induction and after restraint stress. (A) Total number of c-*fos* positive cells counted in sections from naïve (untreated) wild-type, *cacna1a* (R192Q), EP2$^{-/-}$and GlyRα3$^{-/-}$ mice (n = 6 per group). Values are expressed as mean ± SEM from 5 sections per mouse at different levels of the TNC. (B) Number of c-*fos* positive cells counted in sections from saline-treated (control group) wild-type, *cacna1a* (R192Q), EP2$^{-/-}$and GlyRα3$^{-/-}$ mice (n = 7 per group) and from CSD induced wild-type, *cacna1a*(R192Q), EP2$^{-/-}$and GlyRα3$^{-/-}$ mice (n = 8 per group). Values are expressed as mean ± SEM from 6 - 8 sections per mouse at different levels of the TNC. Black bars = ipsilateral TNC, white bars = contralateral TNC. Two way ANOVA (treatment: F = 11.46, p = 0.002; genotype: F = 1.11, p = 0.354; genotype * treatment: F = 2.603, p= 0.063). ** p < 0.01, pairwise comparison. (C) Number of c-*fos* positive cells counted in sections from restrained-stress wild-type, *cacna1a* (R192Q), GlyRα3$^{-/-}$ and *cacna1a* (R192Q)/GlyRα3$^{-/-}$ double mutant (n = 6 per group). Values are expressed as mean ± SEM from 5 sections per mouse at different levels of the TNC.

Analysis of CSD susceptibility

Electrical stimulation

To confirm the predisposition of *cacna1a* (R192Q) mice to CSD (van den Maagdenberg, 2004), we first assessed the susceptibility of these animals to electrically induced CSD compared to wild-type littermates. We analyzed the threshold for initiation of CSD in anaesthetized mice after stimulating the occipital cortex with a bipolar electrode. A CSD event was successfully evoked and recorded in all tested mice. We observed high variability in the values obtained from both *cacna1a* (R192Q) and wild-type littermates (Fig.6). When comparing the two groups, no significant difference was found (333.32 ± 136.62 µA and 316.66 ± 172.24 µA respectively, t-test, p=0.85).

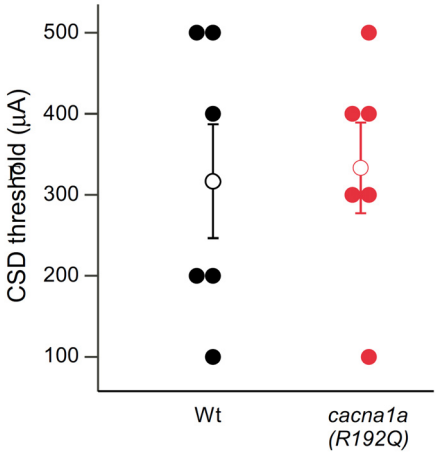

Fig.6 Electrically induced CSD thresholds. Summary of the thresholds required to elicit CSD in wild-type (n=6) and *cacna1a* (R192Q) (n=6). Data are expressed as single values, mean ± standard deviation.

Chemical stimulation

Considering the variability obtained in the electrically induced CSD experiments, we established an alternative method to measure CSD susceptibility using solutions containing increasing concentrations of KCl to induce the change in DC potential. We determined the threshold for CSD occurrence, the spreading velocity and the latency between KCl application and CSD initiation by applying increasing concentrations of KCl onto the occipital cortex of anesthetized mice and measuring changes in the potential at two different cortical sites (parietal and frontal (Fig.7A)). Compared to their wild-type littermates, *cacna1a* (R192Q) mice

showed lowered thresholds for CSD induction (58 ± 4.2 vs 86 ± 5.4 mM, ** $p < 0.01$) (Fig.7B). *Cacna1a* (R192Q) mutant mice also displayed a faster propagation of cortical depression along the postero-anterior axes mice (4.6 ± 0.22 vs 3.3 ± 0.29 mm/min, * $p < 0.05$) (Fig.7C). Moreover, the latency between topical KCl application and the occurrence of cortical depression at the parietal recording site was significantly shorter in *cacna1a* (R192Q) mice compared to wild-type (49 ± 6.3 vs 80 ± 8.3 s, * $p < 0.05$) (Fig.7D).

It is still not clear whether direct and/or indirect pathways connecting the cerebral cortex to the trigeminal system could be responsible in linking CSD and trigeminal activation. Increased susceptibility to CSD is likely to facilitate sensitization of the trigeminal system. To analyze the contribution of the EP2 / GlyRα3 pathway to the cortical phenomena associated with migraine, we have characterized CSD susceptibility in GlyRα3$^{-/-}$, EP2$^{-/-}$ and double mutant mice (mice carrying the point mutation in the cacna1a gene and lacking either the GlyRα3 gene or the EP2 gene). Thresholds, propagation velocities and latencies measured in GlyRα3$^{-/-}$ were not different from wild-type mice (Fig.7B, C and D). Notably, EP2$^{-/-}$ displayed a similar phenotype as the *cacna1a* (R192Q) mice, with a lowered threshold (63.39 ± 1.78 mM, *** $p< 0.001$), an increased propagation rate (4.4 ± 0.18 mm/min, * $p < 0.05$) and a decreased latency (55 ± 8.57 s, * $p < 0.05$) compared to wild-type mice. We also investigated CSD susceptibility in *cacna1a* (R192Q)/ GlyRα3$^{-/-}$ and *cacna1a* (R192Q)/ EP2$^{-/-}$ double mutant mice. In both groups CSD thresholds were significantly reduced compared to wild-type (60 ± 4.7 and 58 ± 5.5 mM, *** $p < 0.001$). Furthermore, both groups of double mutant mice showed a tendency towards decreased latencies between KCl application and CSD onset compared to wild-type mice. Unlike *cacna1a* (R192Q) mice, CSD propagation velocity was similar to wild-type mice.

These results illustrate that *cacna1a* (R192Q) mice are more susceptible to chemically-induced CSD compared to wild-type littermates, confirming previous findings obtained with electrically-induced CSD (van den Maagdenberg *et al.*, 2004). Interestingly, a similar predisposition to chemically-induced CSD was found in mice lacking EP2 receptors but not in GlyRα3 deficient mice. Collectively, these data point to an involvement of the EP2 pathway in the mechanisms conferring susceptibility to chemically-induced spreading depression in the cortex. On the other hand, GlyRα3 mediated glycinergic inhibition does not appear to be

relevant to this phenomenon. Therefore these findings suggest the possibility that other effectors may contribute to the EP2 pathway responsible for the increased chemically-induced CSD susceptibility.

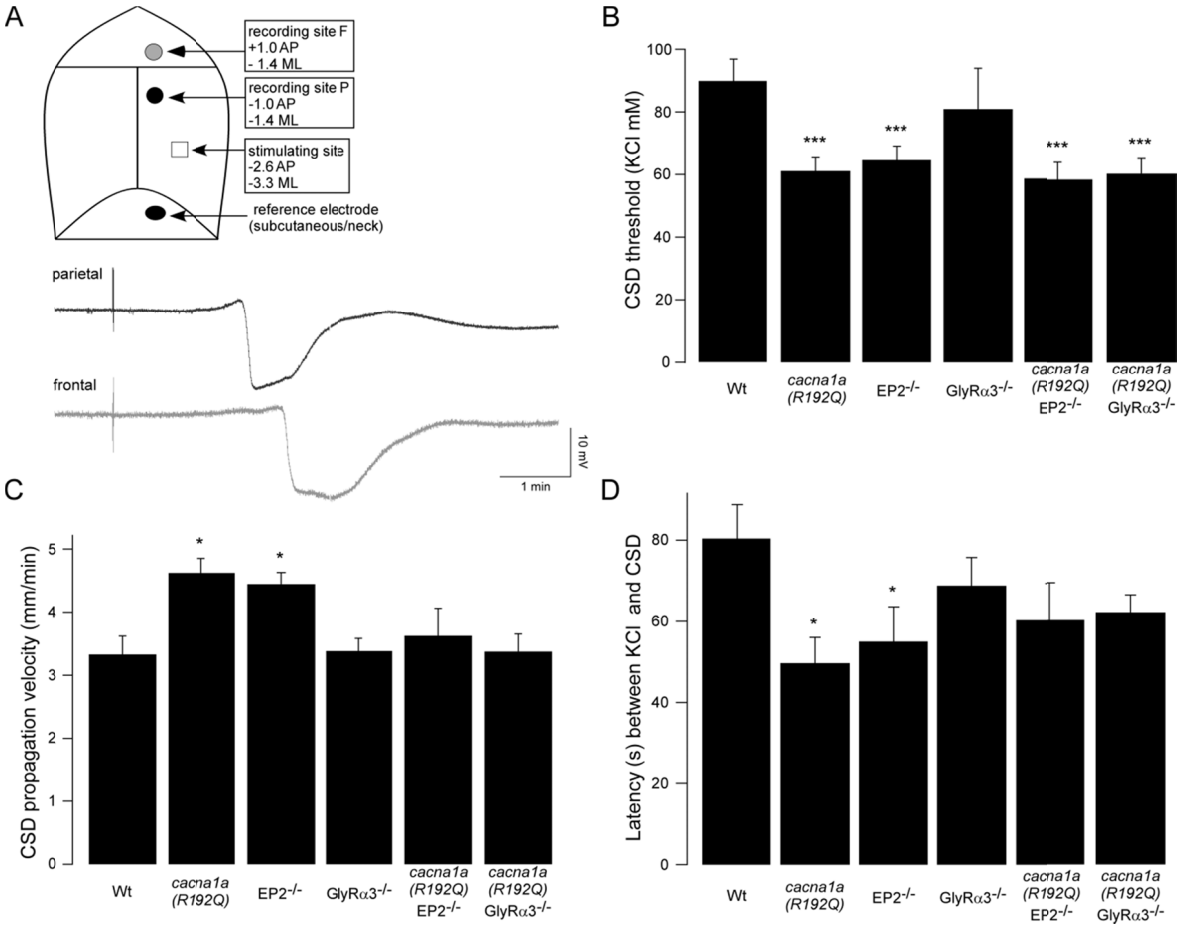

Fig.7 KCl induced CSD susceptibility. (A) Diagram illustrating location of the stimulating and recording electrodes. Examples of the CSD recordings at parietal and frontal recording windows are shown. (B) CSD threshold (KCl concentration, mM) in wild-type (n=15), *cacna1a* (R192Q) (n=12), EP2 $^{-/-}$(n=14), GlyRα3$^{-/-}$ (n=12), *cacna1a* (R192Q)/ GlyRα3 $^{-/-}$ double mutant (n=7) and *cacna1a* R192Q / EP2 $^{-/-}$double mutant mice (n=7). Values are expressed as mean ± SEM. ANOVA followed by Dunnett's post-hoc test, F (5, 66) = 10.61; ***, $P < 0.001$, significantly different from wild-type. (C) CSD propagation velocity (mm/min) in wild-type (n=15), *cacna1a* (R192Q) (n=12), EP2$^{-/-}$ (n=14), GlyRα3$^{-/-}$ (n=12), *cacna1a* R192Q / GlyRα3 $^{-/-}$ double mutant (n=7) and *cacna1a* (R192Q) / EP2 $^{-/-}$double mutant mice (n=7). Values are expressed as mean ± SEM. ANOVA followed by Dunnett's post-hoc test, F (5, 66) = 7.64; *, $P < 0.05$, significantly different from wild-type. (D) Latency between KCl application and onset of CSD at the parietal site in wild-type (n=15), *cacna1a* (R192Q) (n=12), EP2 $^{-/-}$(n=14), GlyRα3$^{-/-}$ (n=12), *cacna1a* (R192Q) / GlyRα3 $^{-/-}$ double mutant (n=7) and *cacna1a* (R192Q) / EP2 $^{-/-}$double mutant mice (n=7). Values are expressed as mean ± SEM. ANOVA followed by Dunnett's post-hoc test, F (5, 66) = 6.87; *, $P < 0.05$, significantly different from wild-type.

Effect of TG6-10-1 (EP2 receptor antagonist) on CSD susceptibility

To further explore the contribution of EP2 receptors and to confirm our findings obtained in EP2 deficient mice, we investigated the effect of pharmacological antagonism of the EP2 receptor on chemically-induced CSD susceptibility. To this end we used a recently developed EP2 receptor antagonist, TG6-10-1. This compound displays a high selectivity for EP2 (Ki=21.4 nM) compared to other Gαs-coupled GPCR receptors (Ki for EP4=13.4 μM, no effect on β2-adrenergic receptors) and has been successfully used to prevent brain damage after seizures in a mouse model of epilepsy (Jiang *et al.*, 2012). We first studied whether a systemic application of TG6-10-1 could affect CSD thresholds. TG6-10-1 did not affect CSD susceptibility when applied intraperitoneally. Wild-type mice treated with TG6-10-1 one hour before CSD induction displayed a similar CSD threshold compared to mice treated with vehicle (81.87± 4.81 vs 79.37 ± 3.41 mM). Moreover, the values obtained in both groups were largely comparable to untreated wild-type mice (Fig. 8).

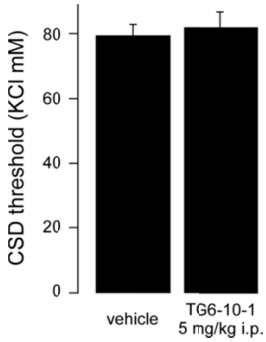

Fig.8 Effect of TG6-10-1 on the susceptibility to CSD after i.p. administration. CSD thresholds (KCl concentration, mM) in vehicle treated mice and in mice treated with TG6-10-1 (5 mg/kg i.p., 10 ml/kg body weight) 1 hour before CSD induction (n=6 mice per group). Values are expressed as mean ± SEM. T-test versus vehicle, p=0.46.

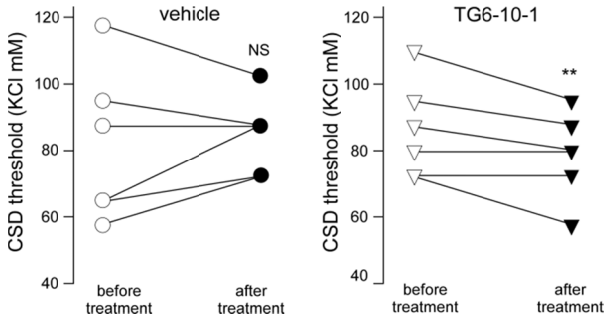

Fig.9 Effect of TG6-10-1 on the susceptibility to CSD after dural application. First (before topical treatment) and second CSD threshold (after topical treatment) (KCl concentration, mM) are shown for each mouse. ○, ● vehicle treated mice; ▽,▼ TG6-10-1 (100 μM, topical dural application) treated mice (n=6 mice per group, ** $p < 0.01$, paired t-test).

Since it is possible that a local activation of EP2 receptors is associated with the increased chemically-induced CSD activation, we next evaluated the effect of TG6-10-1 after topical application on the dura mater. We have compared the thresholds obtained in the first CSD induction to the values collected after the application of vehicle/drug. None of the previously analyzed groups of mice showed a significant difference between the first and the second CSD (e.g. in wild-type mice 83 ± 10.2 vs 82 ± 4.5; n = 12, p = 0.81, paired t-test). Similarly, the application of vehicle in wild-type mice did not affect CSD thresholds after topical treatment (77 ± 6.3 vs 78 ± 3.2; n = 6, p = 0.75, paired t-test). Instead, CSD thresholds measured after dural application of TG6-10-1 were significantly lower when compared to the values obtained before drug treatment (78 ± 4.9 vs 85 ± 6.8, ** $p < 0.01$, paired t-test) (Fig. 9).

These results show that local antagonism of cortical EP2 receptors causes an increase in chemically-induced CSD susceptibility which is in line with the data obtained in EP2 deficient mice. These findings thus suggest that local activation of the EP2 pathway could reduce the cortical susceptibility to spreading depression.

Discussion

In this study, we explored a possible contribution of EP2 receptors and GlyRα3 to the pathophysiology of migraine using the *cacna1a* (R192Q) mice as a reference model (van den Maagdenberg *et al.*, 2004). We can confirm that *cacna1a* (R192Q) mice are more prone to CSD than the corresponding wild-type mice. Indeed, we have shown that *cacna1a* (R192Q) mice possess an increased susceptibility to chemically induced CSD, as revealed by a reduced threshold of CSD activation, increased spreading velocity and reduced delay between KCl application and CSD occurrence. It is likely that the increased cortical excitability observed in our experiments results from a gain of function by Cav2.1 channels associated with the R192Q mutation (Tottene *et al.*, 2009). Despite this increased CSD susceptibility, we were not able to find any behavioral correlates of migraine in *cacna1a* (R192Q). Specifically, *cacna1a* (R192Q) mice failed to show increased photophobia as compared to wild-type littermates. Using c-*fos* expression as a marker of neuronal activation, we also did not find any indication of increased activation of the TNC in *cacna1a* (R192Q) mice. These mice displayed similar levels of c-*fos* activation in the TNC compared to wild-type littermates even after the induction of multiple CSDs. The absence of increased photophobia contrasts to a previous report (Chanda et al., 2008) showing that *cacna1a* (R192Q) mice exhibited increased photophobic behavior in a modified elevated plus maze. Possible explanations for this discrepancy include differences in the exposure of the mice to stressors, which might provoke migraine attacks in *cacna1a* (R192Q) mice. In our experiments we assessed photophobia in the Intellicage through a standardized and automated system in an undisturbed, unrestrained environment and in a social context with minimal disturbance by the experimenter (Mechan *et al.*, 2009).

Next, we explored a possible contribution of EP2 and GlyRα3 receptors to central sensitization. GlyRα3 expression is restricted to the CNS where it is primarily involved in spinal nociceptive processing and brainstem respiratory control (Harvey *et al.*, 2004; Manzke *et al.*, 2010). For the first time, we have shown that GlyRα3 is expressed in the superficial laminae of the TNC, thus suggesting their involvement in the control of excitability at the trigeminal level. The lack of GlyRα3 promoted an increase in c-*fos* activation in the TNC after craniotomy and dural application of saline. These results suggest a role of GlyRα3-mediated

synaptic inhibition in the control of excitability in the TNC after local mechanical injury. On the other hand, GlyRα3$^{-/-}$ mice displayed a similar susceptibility to chemically-induced CSD compared to wild-type mice and did not show any aversion to light in our photophobia experiments. Accordingly, they failed to show a further increase in the number of c-*fos*-positive cells after induction of CSD. Thus, GlyRα3-mediated inhibition does not appear to be important for the control of cortical excitability and light aversion.

Compared to GlyRα3, EP2 receptors are widely expressed in the CNS and in non-neural tissue (Kawabata, 2011). They mediate a large variety of cellular responses ranging from smooth muscle relaxation, over enhancement of inflammatory mediators to regulation of immunoglobulin expression (Breyer *et al.*, 2001). They are abundantly distributed in smooth muscle, in inflammatory cells and in cells of the blood vessel wall where their activation causes vasodilation (Coleman *et al.*, 1994). EP2 receptors are also widely expressed in neurons of forebrain structures as well as of the thalamus, hypothalamus, brainstem, and spinal cord (Andreasson, 2010). The mRNA expression of EP2 receptor has been demonstrated by RT-PCR in rat cranial arteries (middle meningeal and middle cerebral arteries), in the trigeminal ganglion and in the TNC, suggesting a possible role in trigeminal sensitization (Myren *et al.*, 2012). The lack of EP2 receptors did not predispose mice to light aversion. Moreover EP2$^{-/-}$ mice displayed a similar TNC activation compared to wild-type mice, both after simple craniotomy or after multiple CSDs. Surprisingly, EP2$^{-/-}$ mice showed a similar susceptibility to KCl-induced CSD comparable to that seen in *cacna1a* (R192Q) mice. Our findings obtained in EP2$^{-/-}$ mice were confirmed using TG6-10-1, a recently developed EP2 receptor antagonist. This small molecule showed high potency and selectivity at EP2 receptor, with a selectivity index of 626 compared to the EP4 receptor. Administration of TG6-10-1 reduced neuronal injury induced by status epilepticus in mice (Jiang *et al.*, 2012). The application of the EP2 antagonist TG6-10-1 on the dura caused an increase in the susceptibility to chemically-induced CSD in our experiments. However, the pre-emptive systemic administration of TG6-10-1 did not affect the susceptibility to KCl-induced CSD. Whether the increased susceptibility observed after the dural application of the EP2 antagonist comes from vascular EP2 receptors or from EP2 receptors residing on neurons or glia is not known. Further studies with tissue specific conditional, neuron- or endothelial cell

specific $EP2^{-/-}$ mice might help to elucidate the location of the EP2 receptors relevant for this increased CSD susceptibility.

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of migraine attacks (Magis *et al.*, 2011). They mainly act through the inhibition of COX enzymes, thus limiting the production of prostaglandins such as PGE_2, the endogenous activator of EP2 receptors. Our observation that the local blockade of EP2 receptors renders the mice more prone to CSD and potentially predisposes them to migraine attacks. This process may contribute to a disorder called medication overuse headache (MOH), i.e. a syndrome that quite frequently develops in migraineurs after overuse of anti-migraine drugs (De Felice *et al.*, 2011). In particular, the abuse of acute pain medications such as NSAIDs has a major role in this disorder (Diener *et al.*, 2011). In the light of our results, we propose that the recurrent use of NSAIDs could cause chronic alterations in the EP2 receptor activation pathways possibly leading to an increased sensitivity to CSD. Further investigation on the effects of chronic EP2 antagonism on the susceptibility to CSD will help to elucidate the mechanisms involved in medication overuse headache.

The pathophysiology of migraine is still largely unknown and several mechanisms have been proposed. Our data demonstrate that the EP2 receptors do not contribute to central sensitization associated with migraine pain through the previously well described PGE_2/ GlyRα3 signal transduction pathway. Instead our findings suggest an involvement of EP2 receptors in the regulation of cortical excitability.

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2nd Project:

EEG activities in the cortex of migraine mutant mice recorded with Neurologger

Manuscript in preparation.

Abstract

Despite most mechanisms of migraine neurobiology are still unclear, there is strong evidence that visual aura arises from CSD, an electrical phenomenon of the cortex also described in other brain disorders. Several imaging studies have shown CSD-typical changes in the cortex of migraineurs while experiencing a visual aura. Findings from several clinical studies propose that there could be a generalized interictal hyperexcitability of the cerebral cortex in migraine, more pronounced in visual areas because of its neuronal density and possibly favoring the occurrence of CSD. Experimentally evoked CSD can be recorded in anaesthetized mice as a shift in the DC potential. Moreover, evoked CSD can be observed as a flattening in the EEG traces.

Since the occurrence of CSD events changes EEG patterns, EEG recordings in freely moving mice carrying a genetic predisposition to migraine could represent a suitable approach to study spontaneous CSD. Here, we compared the susceptibility to potential spontaneous CSDs of wild-type and *cacna1a* (R192Q) mutant mice in using Neurologger, a cable-free data logging system which allows EEG recording in freely moving mice. Despite their increased susceptibility to evoked CSD, we did not find evidence for events of spontaneous CSD disorders nor for interictal excitability in the EEG of *cacna1a* (R192Q) mutant mice. For future experiments, additional known triggers of migraine such as stress and hunger might be useful to promote the occurrence of spontaneous CSD events.

Introduction

CSD is a self-propagating wave of transient intense depolarization followed by a long-lasting neuronal suppression. Interest in this phenomenon comes from the hypothesis that CSD underlies migraine aura (Tfelt-Hansen, 2010). Moreover, there is strong clinical and experimental evidence to suggest that CSD is involved in the pathophysiology of other disorders such as stroke, subarachnoid hemorrhage and traumatic brain injury (Lauritzen *et al.*, 2011). In humans, CSD has been recorded using subdural electrocorticography (EcoG). A depression of background EcoG activity and a transient change in the DC potential spreading at a slow rate has been shown in patients with ischemic stroke or acute brain injury during craniotomy (Fabricius *et al.*, 2008). Obviously, such invasive interventions are not applicable to migraine patients, but neuroimaging studies during migraine with aura have documented a number of hemodynamic changes including local alterations in cerebral blood flow, cortical hyperemia, spreading oligaemia, and hypoperfusion reflecting CSD (Smith *et al.*, 2008). In animal models, CSD can be experimentally induced by focal mechanical, electrical or chemical stimulation. These types of evoked CSD have been extensively characterized as a shift in the DC potential in anaesthetized mice (Martins-Ferreira *et al.*, 2000). However, the methodological complexity of recording DC potential in the human cortex and in conscious animals significantly limits studies of the functional consequences of CSD.

CSD appears also to alter EEG (electroencephalography) activity patterns (Tfelt-Hansen, 2010). Changes in the amplitude and frequency of the EEG signal normally reflect transitions between different vigilance states. Indeed three vigilance states have been described in mammals: waking, nonREM sleep and REM (Rapid Eye Movement) sleep (Achermann and Borbely, 2011). Wake state is characterized by high theta activity (6-10 Hz in rodents). NonREM sleep is also defined as slow wave sleep since it consists mostly of delta activity with high amplitude waves at less than 3.5 Hz. The EEG in REM sleep appears somewhat similar to the awake EEG. It can be differentiated from the awake EEG by using an EMG (electromyography) electrode that serves for evaluating and recording the electrical activity produced by skeletal muscles. Interestingly, evoked CSD has been shown as a transient and reversible depression in the EEG traces of conscious rabbits (Koroleva *et al.*, 2009a; Leao, 1947). Different studies have in addition shown an increased relative theta activity in

migraineurs. Furthermore, patients with high pain intensities seem to have more delta activity than those with less intense pain. These results suggest that migraine is associated with a mild brain dysfunction between attacks (interictal), possibly caused by activity changes in subcortical or limbic structures (Bjork *et al.*, 2009). Moreover, migraine patients may also possess a generalized interictal hyperexcitability of the cerebral cortex, more pronounced in visual areas because of its neuronal density (Welch *et al.*, 1990) and low content of astrocytes (Largo *et al.*, 1997) , and possibly favoring the occurrence of CSD (Lauritzen, 1992; Schoenen *et al.*, 2003). Migraineurs process sensory stimuli abnormally during and between attacks. Prior studies have demonstrated that migraineurs are hypersensitive to light, sound and odours interictally (Aurora *et al.*, 1999). There is also evidence that migraineurs process somatosensory stimuli abnormally during a migraine attack (Schoenen *et al.*, 2003). Interictal sensitization is accompanied with lower the activation threshold of the trigeminal system, thus possibly predisposing the migraine patient to future migraine attacks. Migraineurs demonstrate a hyper-responsiveness to certain visual stimuli. They have a lower threshold for phosphenes induced by transcranial magnetic stimulation (TMS) of the occipital cortex and visual evoked potentials (VEPs) in migraineurs fail to show the habituation to repetitive presentations of stressful stimuli typically seen in healthy volunteers (Hansen *et al.*, 2011).

At present, most experimental studies record CSD with invasive electrodes placed in the cortex of anaesthetized mice and use mechanical, chemical or electrical stimulation of the cortical surface to evoke CSDs. Recording of spontaneously occurring CSDs in freely moving mice might however much better reflect the natural situation. Since the occurrence of CSD events changes EEG patterns, EEG recordings in freely moving mice carrying a genetic predisposition to migraine could represent a suitable approach to study spontaneous CSD.

Here, we have recorded EEG in conscious and freely moving mice using a cable-free data logging system. To this end, we compared the susceptibility to potential spontaneous CSDs of wild-type and *cacna1a* (R192Q) mutant mice. These mutant mice carry a knock-in mutation (R192Q) in the α1 pore-forming subunit of P/Q type calcium channel ($Ca_v2.1$) which is found in patients suffering from a hereditary form of migraine called familial hemiplegic migraine (FHM) (van den Maagdenberg *et al.*, 2004). In vitro and ex vivo studies

have shown that the mutated calcium channels open at lower voltages. This gain of function results in facilitated excitatory neurotransmission whereas inhibitory neurotransmission remains unaffected (Tottene *et al.*, 2009). At present, *cacna1a* (R192Q) mutant mice represent the best characterized genetic migraine mouse model (Vecchia *et al.*, 2012). In the analysis of the EEG recordings we have first applied a power spectrum analysis to differentiate the three vigilance states by calculating the power of the different frequencies, and then explored the possibility that *cacna1a* (R192Q) mutant mice exhibit an interictal cortical hyperexcitability by analyzing the occurrence of paroxysmal activity in the cortex of mutant mice compared to wild-type littermates.

Materials and Methods

Data-logging device Neurologger. The device called NeuroLogger® (NewBehavior AG) has a weight of 2.8 g including batteries and can be plugged into or removed from a connector embedded in a dental cement socket on the skull of the animal. We used a commercially available version of a prototype system described previously for the use in pigeons (Vyssotski *et al.*, 2006). Briefly, it contains 4 input channels for electric signals, 2 reference channels, 1 channel for a movement sensor, and 1 channel for an infrared receiver. Preamplification, analog-to-digital (AD) conversion (unity gain buffer, AC input range ±750 µV, 1000× gain, analog anti-aliasing band-pass filter ranging from 1 to 400 Hz, ADC resolution 8 or 10 bits), and data storage capacity up to 512 MB, are handled by a microprocessor. The amplifier was operated in the 1 reference channel mode with the 2 reference channels internally connected. Sampling rates can be selected by between 200 and 500 Hz. The runtime of the two batteries (2*1.4V=2.8V standard hearing aid Zink-Air batteries) varies with sampling frequency and quality of supplier from 36 to 72 h. Data can be downloaded offline from the microprocessor to a computer through an USB interface cable. The file format is a binary, proprietary custom format briefly referred as hexadecimal or hex file format. For basic design and circuitry of the NeuroLogger, see Vyssotski et al., 2009. The version described here was commercialized under license from the University of Zürich and differs from the initial prototype in four respects: (i) upper sampling rate is set to 500 Hz to handle a wide EEG-signal range yet allowing recordings over several days, (ii) the logger contains a movement sensor obviating the need for recording electromyograms to document movements, (iii) it is equipped with battery holders for hearing aid batteries in order to avoid soldering of leads to batteries, (iv) electrodes are pre-soldered to pins connecting to the data logger to avoid soldering during implantation.

Surgical procedure. Female mice (8 and 12 weeks) were used in this study. Electrode implantations were done stereotaxically under isoflurane anaesthesia. Six holes were drilled into the skull following precise coordinates from the bregma and midline, respectively (Ch1: AP -1.5, ML 1.2; Ch2: AP +0.5, ML 1.2; Ch3: AP +0.5, ML -1.2; Ch4: AP -1.5, ML -1.2; Ref1 and Ground: AP -3.8, ML 1.2; Ref2: AP -3.8, ML -1.2). Gold-plated miniature screws (diameter 0.9 mm) were positioned through the skull on the dura. Before implantation, each screw was

soldered to a soft insulated copper cable. The other end of each cable was soldered to a pin. An exception to this was the cable of the reference electrode, which was soldered to two parallel pins, one functioning as ground. All cables were connected to one common 7-pin connector. Cables and connector were fixed to the skull of the mouse with dental cement. At the end of surgery, the connector was coupled to a "dummy" logging device, a plastic device built with a similar weight and form as the neurologger. After surgical intervention, animals were housed alone to avoid reciprocal damage at the implantation. Mice were given free access to water and pellet food. Rooms were kept at 23±2°C, with artificial non-reversed 12 h/12 h light–dark cycles.

EEG recordings. Recordings were performed during different time periods according to the running time of the batteries in the Neurologger (from 15 to 60 hours, to avoid further manipulation of mice). Mice were recorded between day 7 and day 21 post-surgery. Logged data in hex format files were imported by a script to Spike2-software (CED, Cambridge, UK) for visual evaluation. In addition conversion programs were developed for Matlab R2009a (The Mathworks Inc, Natick, MA, USA) for converting the data into portable file formats, such as memory mapped files for efficient Matlab processing or EDF files for display and manipulation with EDF-viewer Polyman (free software on www.edfplus.info, (Kemp *et al.*, 2010). For all recordings, a 12 h section (from 18 to 6 am) was extracted and employed for the analysis. EEG recordings from 7 wild-type and 7 *cacna1a* (R192Q) mice were analyzed and compared.

Analysis and Processing of EEG recordings

Fourier frequency filtering. Frequency filtering of the EEG signals was performed by Fourier transformation in frequency space.

Analytical signal. The analytical signal can be used to calculate important parameters like the instantaneous amplitude of the signal (Bracewell, 1986). It is composed by the raw or filtered signal as the real component and an imaginary part derived from the raw or filtered signal by the Hilbert transformation, thus meaning that for each time point the analytical signal is described by a complex number.

Signal amplitude. The modulus or absolute value of the complex numbered analytical signal is the instantaneous signal amplitude and hence proportional to the square root of the

instantaneous power of the EEG signal. Furthermore the positive and negative taken amplitudes are building the envelope of the signal trace.

Long range median filtering. Long range median filtering serves as a non-linear filtering scheme to reduce the large noise fluctuations of EEG signals. It is achieved by three steps for saving resources and computing time using the two Matlab functions median and medfilt1. Before filtering the 1D signal array has to be reshaped into a 2D array of epochs. Then the median for every epoch is calculated. Finally the resulting 1D array is filtered using the 1D median filter medfilt1. The order or support length of the filter in samples is the product of the sampling frequency and the duration in seconds of the range. For vigilance state scoring normally a 84 s and for DC-recordings a 21 s support length is used.

Signal conditioning. Signal conditioning is carried out by analyzing the signal amplitude histograms. Signals were normed by matching the maximum peak of the amplitude histogram. Signal processing was done by epochs with a duration of 4 s for scoring and 2 s or 1 s during spike detection and the analysis of DC-recordings.

Scoring vigilance states. The EEG signal of each channel was analyzed together with the locomotion signal leading to an individual scoring for every channel. Outliers were computed and the corresponding epochs excluded from further processing. For every epoch, locomotion signals were classified according to their amplitudes into low or high amplitudes epochs. The EEG signals were then Fourier transformed by epochs. The epochs of low EMG/locomotion amplitudes were further separated by computing the proportion of the spectral power of the delta and theta band, classifying them into high or low relative delta band power. This division of epochs scores the EEG signals into one of the three vigilance states: wake for high amplitude EMG/locomotion epochs, nonREM for high relative delta power and REM for low relative delta power. Next, the power density spectra were calculated for every vigilance state. A statistical analysis was performed comparing the grand mean of the fractions of vigilance states and the grand mean and standard deviation of the mean spectra.

Analysis of DC recordings. DC signals were recorded according to the method section in project 2**.** DC signals obtained before, during and after KCl induced cortical spreading depression were high pass filtered (Pass band 0.25-45Hz), thus separating the EEG from the

DC part. For visualization and further processing the median of the instantaneous signal amplitude per 1 s epoch was calculated thereby reducing the data for visualization. Finally with medfilt1 of Matlab the median amplitude was filtered using a support length of 21 s needed for reducing large noise fluctuations in order to follow the course of the mean signal amplitude.

Detection of spontaneous cortical spreading depression events. Visual analysis of cortical depressions was supported by automated detection of interregional depression of the EEG signal. This was achieved with instantaneous signal amplitudes by comparing the long range median filter output of two channels with support length of 84 s. This reduces large noise fluctuations and allows following the time course of the mean signal amplitude. For every 4 s-epoch the normed differences were calculated according to the formula: (channel(a) - channel(b)) /(channel(a) + channel(b)). The automated analysis generated a sorted list of normed differences associated with the corresponding time and epoch number.

Spike Analysis. The analysis of spikes was transformed to the task of analyzing the statistical distribution of features characterizing extremal instantaneous signal amplitudes. The normed maxima were used to compare the two different groups of mice. It is the maxima of the amplitude within an epoch (2 s) divided by the corresponding long range (84 s) median filter output. For analysis, the histogram of the normed maxima feature was calculated. First, we compared the values of the most frequent feature (which corresponds to the position of the maximum histogram peak), and second, we analyzed the relative size of the right histogram tail for which the normed maxima values are larger than 3.5.

Results

EEG recordings with Neurologger

Here, we used the cable free, non-telemetric data logging system (NeuroLogger®) to record EEG activity in freely moving mice (Fig.1). This system possesses a passive rolling ball that serves as movement sensor. We have first confirmed the reliability of the movement detector replacing one of the gold screw electrode with an EMG electrode and recording the cerebral activity of a wild-type mouse. The signal obtained via the movement sensor was thoroughly comparable with the one recorded with the EMG electrode (e.g. low EMG corresponds to absence of detected movement and viceversa) (data not shown). We have analyzed and compared EEG recordings from 7 wild-type and 7 *cacna1a* (R192Q) mice. For all recording, a 12 h section (from 18 to 6 am) was extracted and employed for the analysis.

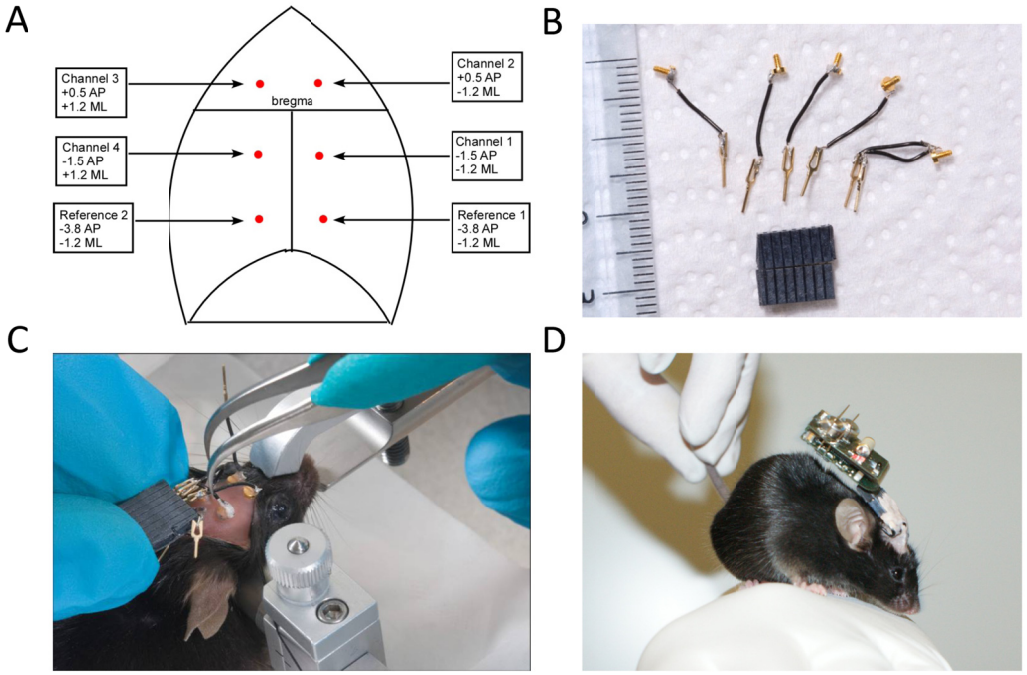

Fig.1 (**A**) Diagram illustrating the position of the screws over the cortex. (**B**) Gold screws were used as EEG-electrodes. Before surgery each screw was soldered to a soft insulated copper cable. The other end of each cable was soldered to a pin. The reference electrode was soldered to two parallel pins, one serving as ground (**C**). All cables were connected to one common 7-pin connector, compatible with the logging device. Cables and connector were all fixated with dental cement. (**D**) A wild-type mouse carrying the Neurologger 2 weeks after implantation.

EEG Spectra

It has been previously shown that chemically-induced CSD in rats alters the EEG patterns during sleep and waking, resulting in an increase in nonREM sleep duration during early recovery from CSD (Faraguna *et al.*, 2010). Here, we have analyzed the impact of the *cacna1a* (R192Q) mutation in mice on sleep patterns. Data were collected from frontal and parietal EEG derivations on both hemispheres.

An automated scoring was performed to differentiate epochs into wake (high movement), non REM sleep (high delta frequencies, low movements) and REM sleep (low delta, low movement). Traces from the different stages possess typical features of the different wake-sleep stage previously recorded with standard cabled EEG (Fig.2).

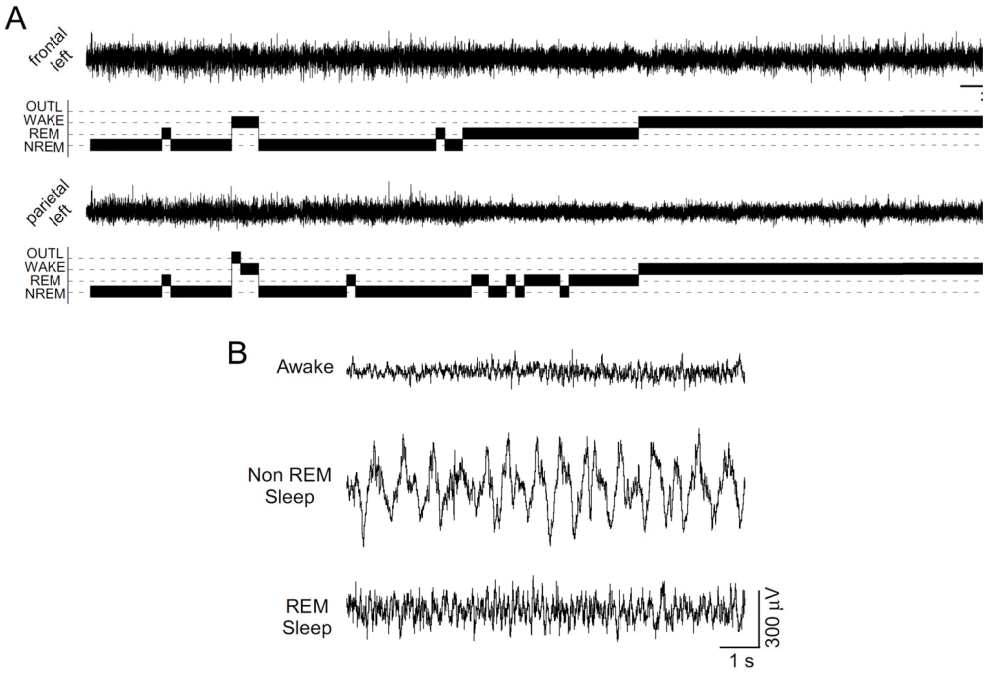

Fig. 2. Representative traces from the EEG (left emisphere frontal and parietal derivates) recorded with Neurologger in a *cacna1a* (R192Q) mouse during waking, nonREM sleep and REM sleep

We have first analyzed the EEG architecture. During the 12 hours interval between 18:00 and 06:00 *cacna1a* (R192Q) mice have been in the average 64% wake and slept for 6% in REM and for 30% non REM sleep. The distribution for WT mice was 65% wake, 4% REM and 31% non REM

The power density obtained for the different sleep stages were in accordance with data normally obtained with cable-based EEG recordings. No differences were observed in the EEG power density in the frequencies between 0 and 20 Hz in all vigilance states in *cacna1a* (R192Q) compared with WT mice (Fig.3A and B). The power spectra graph of a representative wild-type mouse is also shown (Fig.3C). Interestingly, in one of the *cacna1a* (R192Q) mutant mice we have observed an increase EEG power in the non REM sleep in the delta frequencies (Fig.3D)

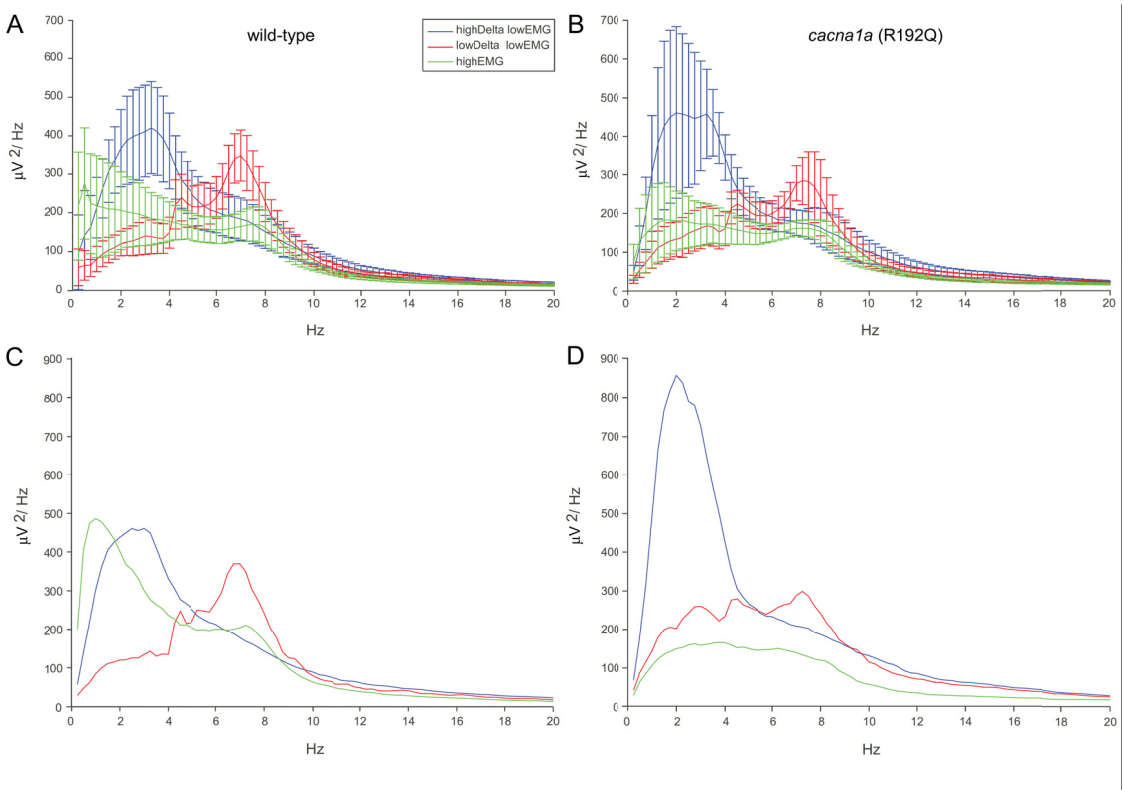

Fig.3. (A) EEG power density in microvolt square per hertz (0.75–20 Hz) in nonREM sleep (high delta, low EMG), REM sleep (low delta, low EMG) , and waking (high EMG) in wild-type mice (*n* = 7) (B) EEG power density in microvolt square per hertz (0.75–25 Hz) in nonREM sleep (high delta, low EMG), REM sleep (low delta, low EMG) , and waking (high EMG) in *cacna1a* (R192Q) (*n* = 7). (C) EEG power density in a representative wild-type mouse. (D) EEG power density in a *cacna1a* (R192Q) mouse who displayed an increase power density in the delta frequencies (2-4 Hz). In all 4 power spectra data refers to channel 3.

Induced EEG depression

As previously described, experimentally induced cortical spreading depression is seen as a transient phase of EEG suppression or desynchronization (Koroleva *et al.*, 2009b; Leao, 1947). We have computed, as described in the methods section, the EEG signal as the high pass frequency part from the DC recording of CSD induction in anaesthetized mice (Fig. 4A, top). The median amplitude of the EEG signal part decreases after the CSD events occur (Fig.4A, bottom). These results confirm that KCl application and subsequent CSD induction cause a decrease in the EEG amplitude in anaesthetized mice.

EEG spontaneous depression

We have searched for the occurrence of spontaneous CSD in the EEG recorded in freely moving mice. We and others have previously shown *that cacna1a* (R192Q) mice display an increase susceptibility to electrically and chemically evoked cortical spreading depression (van den Maagdenberg *et al.*, 2004) and project 1. We speculated that episodes of spontaneous CSD could occur more frequently in *cacna1a* (R192Q) mice than in wild-type littermates. We calculated the normed differences of the median signal amplitudes as described in the method section between the frontal right and the frontal left channels. We have then visually analyzed the recordings according to the sorted output lists of epochs with high normed differences. In only one out of 7 *cacna1a* (R192Q) mice, we found the episodic occurrence of depression of the EEG (Fig. 4B). This phenomenon was not observed in any of the EEG recording from wild-type mice.

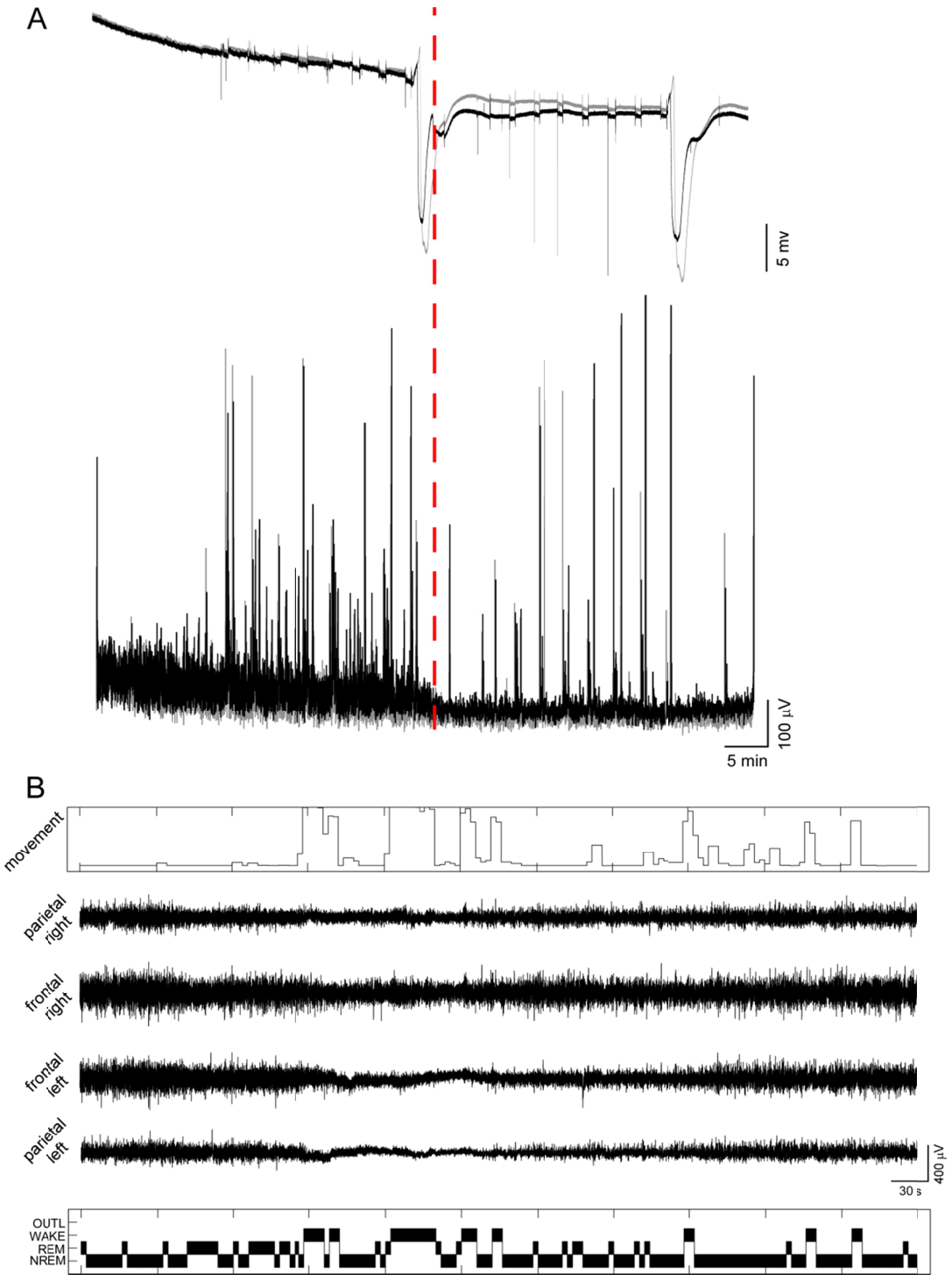

Fig.4. (A) DC traces from parietal (black) and frontal (grey) recording windows. Solutions containing different KCl concentrations were applied to the stimulus site with 7.5 mM step increases at 3 min intervals until a CSD was observed. The protocol was repeated after 5 minutes of wash-out (see Methods of Project 1) (top). Corresponding median amplitudes per 1s epochs. The dashed line indicates the starting of the depression in the signal recorded in both parietal and frontal recording windows that corresponds to the end of the CSD (bottom). (B) Spontaneous EEG depression in a *cacna1a* (R192Q) mouse occurs in all 4 channels.

Interictal spikes

Different studies support the concept of central neuronal hyperexcitability as a critical physiological disturbance predisposing to migraine (Bowyer *et al.,* 2005). Abnormalities in calcium channels physiology could account as potential mechanisms of interictal neuronal excitability (Aurora *et al.*, 2007). From a visual inspection of the different recordings, we have observed the occurrence of spike-like activity in some of the *cacna1a* (R192Q) mutant mice (Fig.5A). As described in the methods section, we have performed an automated analysis of the EEG recordings to measure with a standardized approach the occurrence of spikes. Comparing the normed maximum peak values, we did not find any differences between wild-type and *cacna1a* (R192Q) mice. Moreover the right fraction of the histograms of the normed maxima (right tail, values >3.5) did not differ in the two groups of mice, neither comparing the entire recordings (Fig.5C) nor the fractions characterized by low movement (with less artifacts expected) (Fig.5D).

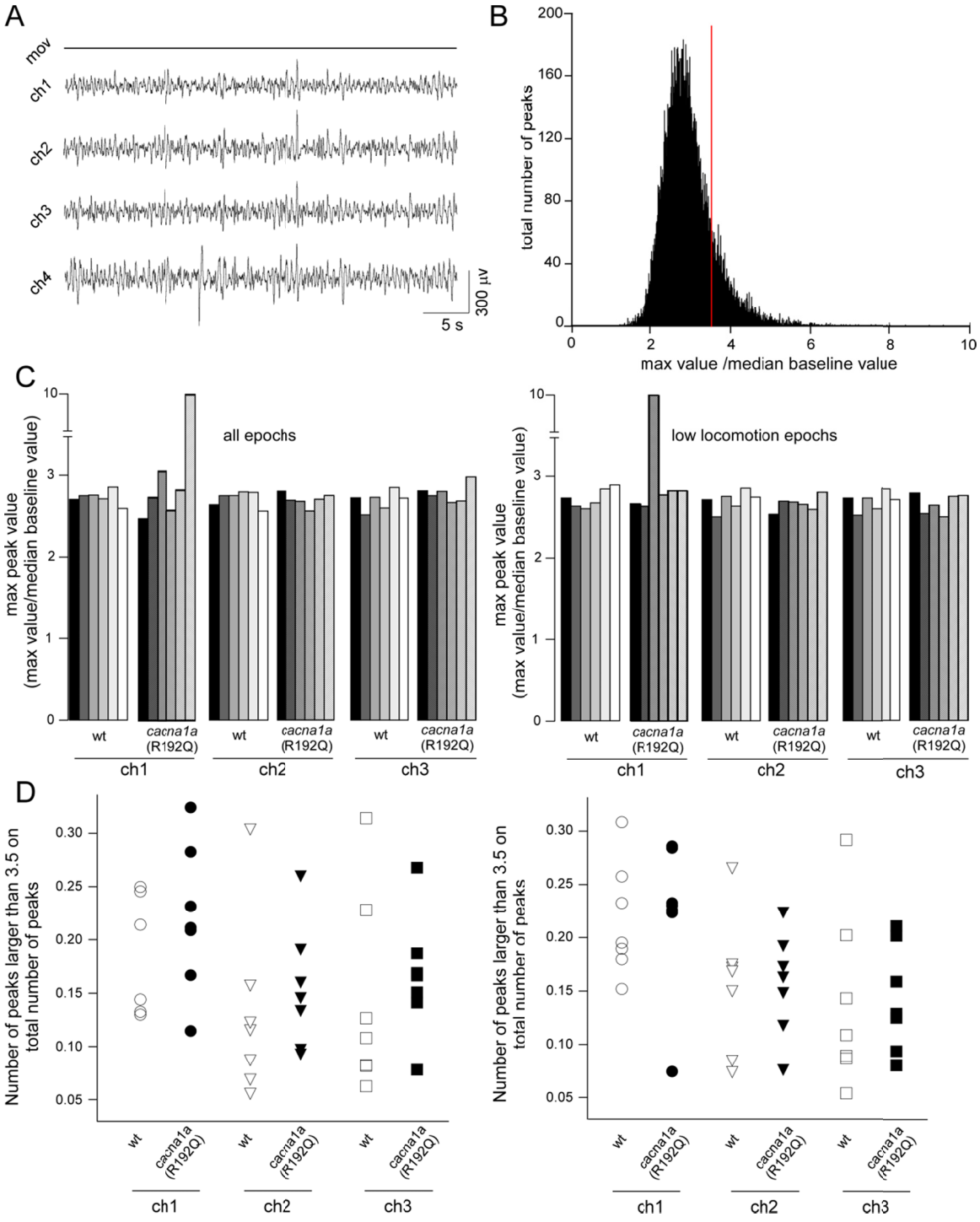

Fig. 5.(A) Representative trace from a *cacna1a* (R192Q) mutant mouse during resting phase where a presumed spike-like activity is noticeable. (B) Histogram of the total number of peaks calculated as the maximum signal amplitudes of 2s epochs compared to the long range (84s) median values (representative example for one *cacna1a* (R192Q) mouse).
(C) Maximum peak values for each mouse and each channels after analysis of all epochs (left) or of low locomotion epochs (right). (D) Fraction of peaks with a maximum value/median value > 3.5 (portion of peak after the line in fig. 5B) after analysis of all epochs (left) or of low locomotion epochs (right).

Discussion

Here, we have used Neurologger, a recently developed cable free data-logging device allowing EEG recordings in freely moving mice (Vyssotski *et al.*, 2006) to evaluate the occurrence of spontaneous CSDs, potential interictal excitability and effects of CSD on sleep patterns in *cacna1a* (R192Q) mutant mice.

Power spectrum analysis demonstrated that *cacna1a* (R192Q) mutant mice and C57Bl/6 wild-type littermates showed similar average durations of waking, nonREM and REM sleep. The distributions of the three different vigilance states were comparable to those of wild-type mice previously described in other studies (Huber *et al.*, 2000; Vyazovskiy *et al.*, 2004). *cacna1a* (R192Q) mutant mice showed in general a higher variability of the vigilance distribution compared to wild-type littermates and one *cacna1a* (R192Q) mutant mouse exhibited a prominent increase SWA peak and prolonged duration of nonREM sleep. In another very recent study, recordings with standard cable-connected EEG showed that under baseline conditions *cacna1a* (R192Q) mutant mice were awake for longer times than wild-type mice and had a reduced amount of nonREM sleep (Deboer *et al.*, in press). Another study addressed the effect of CSD on sleep patterns. This study used KCl-evoked CSDs in awake C57Bl/6 wild-type mice and found altered EEG patterns during sleep and waking with an increase in the slow wave activity (SWA) and in the duration of NREM sleep after CSD (Faraguna *et al.*, 2010). It is important to mention that our analysis was performed based on 12 hour recordings starting at 6 p.m., thus covering almost exclusively the dark period during which mice show only relatively little sleep, while the other studies used 24 hour recordings.

Since the R192Q mutation in the $Ca_v2.1$ channels renders mice more susceptible to evoked chemical and electrical CSD it is plausible to speculate that spontaneous CSD events should also occur more frequently in *cacna1a* (R192Q) mice than in wild-type littermates. Interestingly, we found that application of KCl onto the dura of anaesthetized mice (both *cacna1a* (R192Q) mutant and wild-type littermates) not only induced a depolarizing shift in the DC current but also a decrease in EEG amplitudes, computed from the same recording. This finding confirms that evoked CSD has an effect on the EEG signal also under our experimental conditions in anaesthetized mice. To detect spontaneous CSDs, we have

studied the occurrence of episodic depression of the EEG signal in freely moving mice. Only one out of 7 mutant mice displayed sporadic events of EEG flattening. A possible reason for the absence of spontaneous CSDs in *cacna1a* (R192Q) mutant mice could be that the impact of the R192Q amino acid exchange is less severe in mice than in humans. Such an interpretation is supported by the observation that a significant phenotype (increased CSD susceptibility) was seen in homozygous R192Q mouse mutants only (van den Maagdenberg *et al.*, 2004), while all known human patients are heterozygous carriers of the mutation (Ducros *et al.*, 2001).

Although we did not find significant differences in EEGs of wild-type and *cacna1a* (R192Q) mice, it was still intriguing to see that one mutant mouse behaved differently. Parallel EEG and video recordings may in the future allow a deeper and more systematic analysis and comparison of the EEG signals and the corresponding behavior of the mice. Such combined recordings may also help to evaluate whether changes in the EEG amplitude reflect CSDs or occur as a consequence of an altered vigilance state (desynchronization).

Many studies have described a significant association (comorbidity) of migraine and epilepsy (Ottman *et al.*, 1994). Although these two diseases have distinct clinical manifestations, they are both episodic brain disorders of paroxysmal nature and may share cortical hyperexcitability as a common underlying mechanism. Such a common mechanism may also explain why some antiepileptic drugs are efficacious in both conditions (Rogawski *et al.*, 2008). On mechanistic grounds the observed comorbidity may in many cases originate for example from genetic factors predisposing to both migraine and epilepsy (Ottman *et al.*, 1996). In fact, some autosomal dominant mutations in the *CACNA1A* gene different from the R198Q amino acid exchange are associated with episodic and progressive forms of cerebellar ataxia, vertigo and epilepsy (Beauvais *et al.*, 2004; Kors *et al.*, 2004). Mutations in the *CACNA1A* gene cause a continuum of brain disorders spanning from epilepsy to migraine and the functional consequences of the specific mutation may determine the actual clinical manifestations. In epileptic patients, cortical hyperexcitability manifests itself as interictal spike activity (i.e. activity present between seizures) or spike activity preceding seizures (de Curtis *et al.*, 2001). Interestingly, such abnormal spike activity has also been detected in the EEG of animal models of epilepsy (Glasscock *et al.*, 2007). A preliminary visual examination

of our EEG recordings in *cacna1a* (R192Q) mutant mice recordings had in fact suggested the presence of spike-like activity. Using an objective automated detection system, we did however not find evidence for spike-like activity in *cacna1a* (R192Q) mutant mice. EEG events with features suggestive of interictal spikes were observed with similar frequencies in *cacna1a* (R192Q) mutant mice and in wild type mice. It is therefore likely that these events were movement artifacts in the freely behaving mice.

In summary, our findings support the concept that mice carrying the R192Q mutation, albeit displaying an increased susceptibility to evoked CSD (van den Maagdenberg *et al.*, 2004) do not suffer from spontaneous episodic CSD disorders (spontaneous CSDs) and do not present sign of interictal hyperexcitability in the EEG. Future experiments could be performed that promote the occurrence of spontaneous migraine attacks by using additional known triggers of migraine such as stress and hunger (Chabriat *et al.*, 1999; Martin, 2010). In this context, it might also be interesting to analyze mice carrying another amino acid exchange (S218L), which causes a stronger gain of function effect than the R192Q mutation and a more severe disease in human patients (van den Maagdenberg *et al.*, 2010).

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3rd Project:

HZ166, a Novel $GABA_A$ Receptor Subtype-Selective Benzodiazepine Site Ligand, Is Antihyperalgesic in Mouse Models of Inflammatory and Neuropathic Pain.

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Key words: γ-aminobutyric acid, dorsal horn, disinhibition, hyperalgesia, chronic constriction injury

Published in Neuropharmacology (2011) March; 60(4):626-32.

Presented in poster form at the following conferences:

· FENS (Federation of European Neurosciences), Amsterdam, July 2010

· IASP (International Association for the study of Pain), Montreal, August 2010

· SSPT meeting, Zürich, April 2011

· Society for Neuroscience (SfN, Washington), November 2011

Abstract

Diminished GABAergic and glycinergic inhibition in the spinal dorsal horn contributes significantly to chronic pain of different origins. Accordingly, pharmacological facilitation of GABAergic inhibition by spinal benzodiazepines (BDZs) has been shown to reverse pathological pain in animals as well as in human patients. Previous studies in $GABA_A$ receptor point mutated mice have demonstrated that the spinal antihyperalgesic effect of classical BDZs is mainly mediated by $GABA_A$ receptors containing the α2 subunit (α2-$GABA_A$ receptors), while α1-$GABA_A$ receptors, which mediate the sedative effects, do not contribute. Here, we investigated the potential analgesic profile of HZ166, a new partial BDZ-site agonist with preferential activity at α2- and α3-$GABA_A$ receptors. HZ166 showed a dose-dependent antihyperalgesic effect in mouse models of neuropathic and inflammatory pain, triggered by chronic constriction injury (CCI) of the sciatic nerve and by subcutaneous injection of the yeast extract zymosan A, respectively. This antihyperalgesic activity was antagonized by flumazenil and hence mediated via the BDZ-binding site of $GABA_A$ receptors. A central site of action of HZ166 was consistent with its pharmacokinetics in the CNS. When non-sedative doses of HZ166 and gabapentin, a drug widely used in the clinical management of neuropathic pain, were compared, the efficacies of both drugs against CCI-induced pain were similar. At doses producing already maximal antihyperalgesia, HZ166 was devoid of sedation and motor impairment, and showed no loss of analgesic activity during a 9-day chronic treatment period (i.e. no tolerance development). These findings provide further evidence that compounds selective for α2- and α3-$GABA_A$ receptors might constitute a novel class of analgesics suitable for the treatment of chronic pain.

Introduction

Diminished pain control by glycinergic and GABAergic neurons in the spinal cord is a major contributing factor to chronic pain of inflammatory and neuropathic origin (Zeilhofer, 2008). Restoring synaptic inhibition should therefore be a rational strategy for the treatment of such conditions. Previous work from different groups has shown that local spinal (intrathecal) or systemic application of BDZ-site agonists alleviates inflammatory or neuropathic pain in animals (Knabl *et al.*, 2008; Knabl *et al.*, 2009; Kontinen *et al.*, 2000), and labor pain in human patients (Tucker *et al.*, 2004). However, the wide-spread expression of $GABA_A$ receptors throughout the CNS and various central side effects including sedation, memory impairment, and addiction strictly limit or even preclude the use of classical BDZs in chronic pain patients. Advances in our understanding of the molecular diversity of $GABA_A$ receptors have raised hopes that a separation of desired and undesired actions of classical BDZs could become possible through the development of subtype-selective or partial BDZ-site agonists (Munro *et al.*, 2009; Zeilhofer *et al.*, 2009a; Zeilhofer *et al.*, 2009b). BDZ-sensitive $GABA_A$ receptors contain at least one of the following α subunits α1, α2, α3 or α5, together with a β subunit and a γ2 subunit in a 2:2:1 stoichiometry. Work in $GABA_A$ receptor point-mutated mice, in which the different subtypes of α subunits have been rendered diazepam-insensitive, has shown that the sedative action of BDZs is mediated by $GABA_A$ receptors containing an α1 subunit (α1-$GABA_A$ receptor), whereas α2-$GABA_A$ receptors were found to be responsible for the anxiolytic properties of classical BDZs. Using these $GABA_A$ receptor point-mutated mice, we could recently demonstrate that α2- or α3-$GABA_A$ receptors are largely responsible for the spinal anti-hyperalgesic actions of classical BDZs, while α1-$GABA_A$ receptors do not contribute. Conversely, pronounced analgesia against formalin-induced pain has also been observed after systemic treatment with diazepam in α1-$GABA_A$ receptor point-mutated mice, which are protected from the sedative effects of diazepam. These results suggest that α1 sparing (non-sedative) BDZ-site agonists should exert a genuine analgesic effect after systemic treatment (Knabl *et al.*, 2009).

In rats, we have previously tested such a non-sedative BDZ-site ligand, L-838,417, which has been developed in the quest for non-sedative anxiolytics (McKernan *et al.*, 2000). L-838,417 showed good antihyperalgesic activity in a rat neuropathic pain model without losing efficacy after repeated treatment (Knabl *et al.*, 2008). However, this compound possesses poor pharmacokinetics in mice with very low bioavailability and very short half-life (Scott-Stevens *et al.*, 2005). Recently, a class of novel 8-substituted triazolo- and imidazobenzodiazepines has been synthesized with the aim to develop novel anticonvulsant BDZ-site ligands with a better side effect profile (Rivas *et al.*, 2009). One of these compounds (HZ166, ligand 2 in (Rivas *et al.*, 2009)) is a non-sedative partial BDZ-site agonist with preferential activity at α2- and α3-$GABA_A$ receptors. It exhibits good anticonvulsive activity at non-sedative doses with minimal toxicity and suitable pharmacokinetics in mice and rats after intraperitoneal (i.p.) application. Here, we have evaluated potential antihyperalgesic effects of HZ166 in mouse models of neuropathic and inflammatory pain, and compared these effects with those of gabapentin, a drug frequently used in neuropathic pain patients.

Materials and Methods

Drugs. HZ166 was synthesized as described previously (Cook *et al.*, 2006). Flumazenil was purchased from Tocris Bioscience. For i.p. injection HZ166, flumazenil and gabapentin (Neurontin®) were suspended in 0.5% methyl cellulose and 0.9% NaCl and applied in a total volume of 10 ml / kg body weight.

Preparation of crude brain and spinal cord membranes. Following decapitation of male C57BL/6 mice (25-30 g), brain and spinal cord were rapidly removed, frozen on dry ice and stored at -80°C. For preparation of crude membranes, the tissue was thawed and homogenized in 10 volumes of 10 mM Tris pH 7.4, 150 mM NaCl, protease inhibitor cocktail (complete Mini, Roche Applied Science). After centrifugation at 1000 g for 10 min at 4°C the supernatant was carefully removed and centrifuged again for 20 min at 20,000 g at 4°C. The pellet containing the crude membranes was re-suspended in 10 mM Tris-HCl pH 7.4, 100 mM KCl, protease inhibitor cocktail, washed once by centrifugation and re-suspension and subjected to the [^{3}H]flumazenil binding assay.

Cell culture. L(-tk) cell lines stably expressing either the α1β3γ2, α2β3γ2, α3β3γ2 or α5β3γ2 subunit combination were kindly provided by K. Wafford, Merk Sharp & Dohme. Cells were cultured in DMEM / 10% FBS, and receptor expression was induced by addition of 1 μM dexamethasone (final concentration). Three to four days after induction of receptor expression, cells were harvested by scraping in PBS and stored at -80°C. For [^{3}H]flumazenil binding, cells were thawed, re-suspended in 10 mM Tris-HCl pH 7.4, 100 mM KCl, protease inhibitor cocktail and homogenized by sonication. After centrifugation at 100,000 g for 15 min at 4°C the pellet was re-suspended in buffer and used for radioligand binding.

[^{3}H]flumazenil binding assay. Aliquots of the crude membranes derived from brain and spinal cord (~50 μg protein) or aliquots of homogenates prepared from L cells expressing the α1β3γ2, α2β3γ2, α3β3γ2 or α5β3γ2 subunit combination (~200 μg protein) were incubated with increasing concentrations of HZ166 and 1.2 nM [^{3}H]flumazenil (87 Ci/mmol, PerkinElmer) in a total volume of 200 μl for 90 minutes on ice. The incubation was terminated by rapid vacuum filtration onto glass fiber filters and washed with ice-cold incubation buffer (10 mM Tris-HCl pH 7.4, 100 mM KCl). Non-specific [^{3}H]flumazenil binding was determined using 10 μM clonazepam. Radioactivity retained on the filters was determined by liquid scintillation counting using a Tricarb 2500 liquid scintillation analyzer. Binding data were analyzed using the GraphPad Prism software (version 5.02, GraphPad Software, USA).

Pharmacokinetics of HZ166 in the brain. The concentrations of HZ166 were measured in the brain at 0.5, 1, 1.5, 2, 4, 8, and 24 hours after intraperitoneal injection of HZ166 (48 mg/kg body weight) in CCI operated mice. Three mice were used for each time point. In these mice, mechanical sensitivity was measured for 5 min immediately preceding killing the mice.

Analytical method. Concentrations of HZ166 in brain tissue were determined using liquid chromatography coupled to an ion trap mass spectrometer equipped with an electrospray source (Esquire 3000, Bruker Daltonics, Billerica, MA, USA). Quantification was performed in MS-MS mode using the following transitions: 357.1→329.1 for HZ-166 and 330.1→295.1 for midazolam-d4. Brains were weighted and homogenized in 2 ml of deionized water. The final volume was adjusted to 5 ml with water. 100 μl of internal standard (midazolam-d4,

500ng/ml) were added to 1 ml brain homogenate samples, and extraction was performed using Oasis® HLB SPE columns. The samples were loaded and cartridges washed with 1 ml of formic acid 0.1%-acetonitrile (85-15 v/v). The cartridges were dried under vacuum. Compounds of interest were eluted with 1 ml of methanol. After evaporation, residues were reconstituted in 100 µl of formic acid 0.1%-acetonitrile (80-20 v/v) and 20 µl were injected onto the HPLC system. Separation was carried out in C18 XTerra® column (5µm x 2,1mm x 50mm, Waters Company, USA). Along with the unknown samples, QC and standards samples, prepared using blank brain spiked with HZ166, covering the expected concentration range were processed.

In vivo studies. Behavioral experiments were performed in 7 – 12 weeks old male mice kept at a 12 / 12 h light / dark cycle with free access to food and water. Permission for the animal experiments has been obtained from the Veterinäramt des Kantons Zürich (ref. no. 121/2006 and 135/2009). All efforts were made to minimize animal suffering. In all behavioral tests, the observer was blinded to the drug treatment.

Pain tests

Neuropathic pain. HZ166 and gabapentin were analyzed in the chronic constriction injury (CCI) model. Unilateral constriction injury of the left sciatic nerve just proximal to the trifurcation was performed as described previously (Bennett *et al.*, 1988; Hosl *et al.*, 2006). Anesthesia was induced and maintained by 2% isoflurane, combined with oxygen (30%). The sciatic nerve was exposed at the mid-thigh level proximal to the sciatic trifurcation by blunt dissection through the biceps femoris muscle. 5 ± 7 mm of nerve were freed of adhering tissue and three chromic gut ligatures (4/0) (Ethicon) were loosely put around the nerve with about 1 mm spacing. The ligatures were tied until they elicited a brief twitch in the hindlimb. The surgical wound was closed in layers. Heat hyperalgesia and mechanical sensitization were assessed 7 - 16 days after surgery.

Inflammatory pain. Inflammatory pain was studied in the zymosan A model (Depner *et al.*, 2003; Meller *et al.*, 1997). 0.06 mg zymosan A (Sigma Chemicals) suspended in 20 µl 0.9% NaCl was injected subcutaneously into the plantar side of the left hindpaw. Mechanical sensitization was assessed 48 hours after induction of inflammation.

Heat hyperalgesia. Paw withdrawal latencies upon exposure to a defined radiant heat stimulus were measured using a commercially available apparatus (Plantar Test, Ugo Basile, Comerio, Italy). 4 - 5 measurements were taken in each animal for every time point and averaged. Measurements of paw withdrawal latencies of the inflamed or injured paw and of the contralateral paw were made alternately.

Mechanical sensitization. Mechanical sensitivity was assessed with dynamic von Frey filaments (IITC, Woodland Hills, CA). 4 - 5 measurements were made for each time point and animal and averaged. Measurements of paw withdrawal thresholds of the injured paw and of the contralateral paw were made alternately.

Tolerance development. Possible development of tolerance against the antihyperalgesic effect of HZ166 was investigated in the CCI model. Starting from day 7 after CCI surgery, HZ166 at the dose of 16 mg/kg or vehicle were administered intraperitoneally once daily for 9 consecutive days. On day 10, each group (vehicle and HZ166) was subdivided in 2 subgroups: one received vehicle and the other HZ166 at the dose of 16 mg / kg. After the last injection, mechanical sensitivity was assessed for 3 hours.

Motor impairment. A possible impairment of motor function was analyzed in the rotarod test. Mice were trained on the rotarod (diameter 3 cm, 2 rpm) with 2 different training sessions in 2 consecutive days. Animals capable of remaining on the rotarod in the absence of treatment for at least 2 min were selected for drug testing. On the test day, the latency to fall off the rod was recorded before and 60 minutes after treatment with vehicle, HZ166 or gabapentin.

Locomotor activity. Mice were tested during the light phase of the day–night cycle. 60 minutes after the intraperitoneal administration of vehicle, gabapentin or HZ166, they were placed in individual circular enclosures (diameter 20 cm), equipped with 4 photocells. Motor activity, expressed as the total number of photocell interruptions, was recorded for 1 h.

Statistical Analyses. Drug effects were expressed as per cent maximum possible effect (MPE) calculated from comparisons of paw withdrawal latencies or thresholds obtained prior to surgery or inflammation and before and after drug treatment. Baseline paw withdrawal thresholds / latencies on day 7 after nerve ligation or on day 2 after zymosan A

injection were subtracted from all later withdrawal thresholds and the area under the curve (AUC) was calculated. ED_{50} values were calculated by fitting the experimental data to Hill's equation with a Hill coefficient (nH) = 1. Unless otherwise indicated, statistical comparisons were made with one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test. *P* values < 0.05 were considered significant.

Results

Affinity of HZ166 for native and recombinant $GABA_A$ receptors

The interaction between HZ166 and native $GABA_A$ receptors was analyzed by competition assays using [^{3}H]flumazenil as radioligand. HZ166 displayed a slightly higher affinity for $GABA_A$ receptors expressed in membranes derived from spinal cord (K_i = 189 ± 10 nM) than in membranes prepared from brain (K_i = 282 ± 6 nM) (Fig 1A).

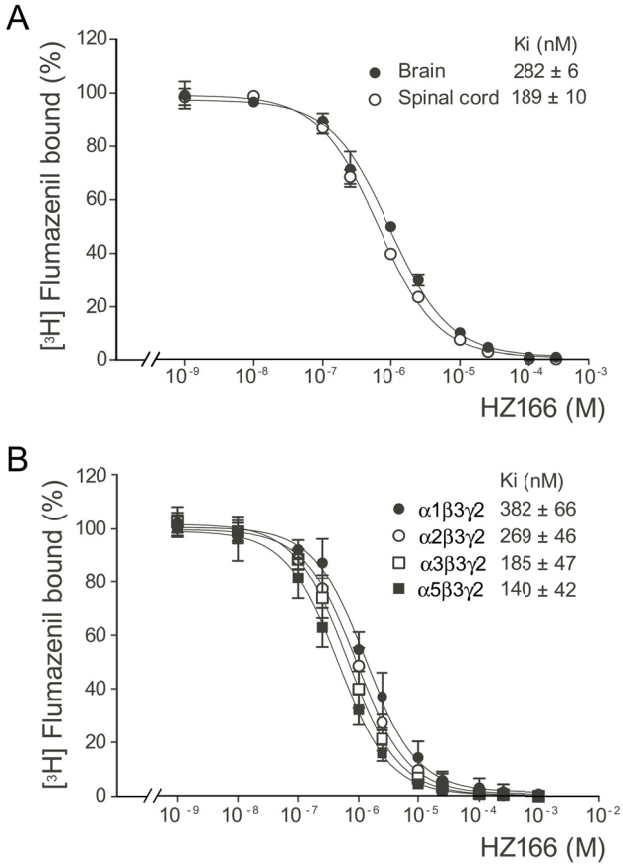

Fig. 1 Affinity of HZ166 for native and recombinant $GABA_A$ receptors. (A) Effects of HZ166 on the binding of [^{3}H]flumazenil to mouse brain (●) and spinal cord (○) membranes. Data represent the means ± standard deviation of 3 independent measurements. Error bars smaller than the symbol are not depicted. **(B)** Effects of HZ166 on the binding of [^{3}H]flumazenil to homogenates prepared from L cells stably transfected with the α1β2γ2, α2β3γ2, α3β3γ2 or α5β3γ2 subunit combination. Data represent the means ± standard deviation of 4 - 5 independent measurements. Error bars smaller than the symbol are not depicted.

Since α1 subunit containing receptors are expressed at higher levels in brain than in spinal cord this result may indicate a preference of HZ166 for $GABA_A$ receptors containing the α2, α3 and α5 subunit. Therefore, the affinity of HZ166 to individual $GABA_A$ receptor subtypes was determined using L cells stably expressing the subunit combinations α1β3γ2, α2β3γ2, α3β3γ2 or α5β3γ2. As expected, HZ166 displayed a statistically significant ($p < 0.05$, one way ANOVA, Bonferroni posttest) higher affinity for receptors not containing the α1 subunit with a rank order of α5 (K_i = 140 ± 42 nM) > α2 (K_i = 269 ± 46 nM) > α1 (K_i = 382 ± 66 nM) (Fig. 1B). The K_i value of HZ166 for the α3β3γ2 combination (185 ± 47 nM) was statistically significantly lower than the K_i value observed for α1β3γ2 but not different from those of α2β3γ2 and α5β3γ2.

Antihyperalgesic effects of HZ166 in neuropathic mice

Antihyperalgesic actions of HZ166 were evaluated in mice, which had undergone chronic constriction injury (CCI) surgery of the left sciatic nerve. Following surgery, operated mice developed progressive behavioral signs of mechanical sensitization (quantified as a decrease in the paw withdrawal threshold [PWT] in response to stimulation with von Frey filaments) and of thermal hyperalgesia (quantified as a decrease in the paw withdrawal latency [PWL] in response to a radiant heat stimulus). Mechanical PWTs and thermal PWLs decreased from 3.45 ± 0.04 g and 16.28 ± 1.23 s pre-surgery to 1.43 ± 0.02 g and 4.67 ± 0.53 s, respectively (mean ± sem, n = 6).

On day 7 after surgery, when sensitization of the ipsilateral paw had reached a plateau, HZ166 was administered i.p. and mechanical sensitivities of the ipsi- and contralateral paws were assessed for 4 hours at 20 min intervals (Fig. 2A). HZ166 significantly increased ipsilateral PWTs in a dose-dependent manner with a maximum effect about 1 h after injection. To quantify the analgesic effects of HZ166, the maximum possible effect (MPE) at 1 hr after injection was calculated for each mouse. Statistically significant antihyperalgesic effects were obtained for doses ≥ 5 mg / kg. Data were fitted to the Hill equation and yielded to an ED50 of 4.8 ± 0.32. PWTs of the contralateral paw were not affected (compare Fig. 2A).

For the dose of 48 mg / kg, we analyzed the concentrations of HZ166 in the brain and compared them with their antihyperalgesic effect in the CCI model over time (Fig. 2B). HZ166 rapidly appeared in the brain (peak value ≤ 0.5 h), demonstrating rapid distribution and ready penetration of the blood–brain barrier. Concentration profiles showed a rapid initial elimination phase for both molecules with an estimated α half-life of 0.39 h for HZ166. A slower β-phase was then observed with an estimated apparent terminal half-life of 6.6 h. The initial estimated α half-life fitted well with the time course of the effect, after a single dose administration (Fig. 2B).

The effect of systemic HZ166 against heat hyperalgesia was assessed in the plantar test (Fig. 2C). Here, we tested a dose of 16 mg / kg, which was the lowest effective dose against mechanical hyperalgesia. HZ166 was administered 7 days after surgery, when the PWL of the CCI-lesioned ipsilateral paw (ipsi) was stable and significantly lower (pre-surgery) than that of the contralateral, non-lesioned paw. HZ166 significantly increased PWLs with a peak effect at 1 hr after the administration. The AUC calculated for the injured paw (4.33 ± 1.95 s * h; n = 6) was significantly different from the vehicle (1.18 ± 0.89 s * h; n = 6; $P < 0.05$, unpaired t-test).

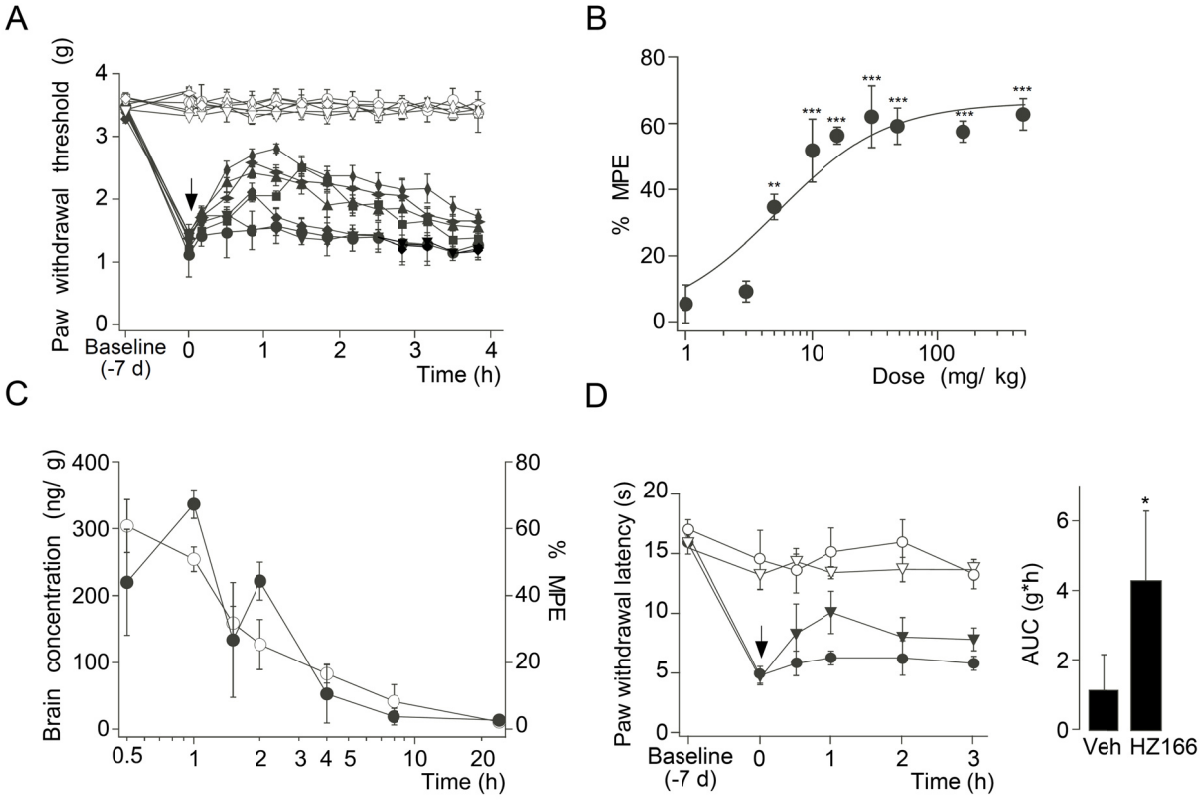

Fig. 2 Antihyperalgesic effects of HZ166 in neuropathic mice. (**A**) Mechanical sensitization. PWTs (g, mean ± SEM) in response to mechanical stimulation with dynamic von Frey filaments were measured before CCI surgery (baseline), on day 7 after surgery before drug administration and for 4 hours after drug injection. HZ166 (1 [▼, ▽], 5 [♦, ◇], 16 [■, □], 48 [▲, Δ], 160 [◂▸, ◇], 480 [⧫, ◊] mg / kg body weight) or vehicle (●, ○) were injected i.p.. Filled symbols, ipsilateral paw; open symbols, contralateral paw. n = 6 mice per group. (**B**) statistical analysis. % MPE, mean ± SEM. ANOVA followed by Scheffe's post hoc test, F (6, 35) = 36.88; $P < 0.001$ *, $P < 0.05$; ***, $P < 0.001$. (**C**) Brain concentrations of HZ166 (○, left axis) and antihyperalgesic effects on mechanical hyperalgesia (●, right axis) were measured after the i.p. administration of 48 mg / kg. Data are expressed as mean ± SEM. n = 3 mice per time point. (**D**) Thermal hyperalgesia. PWLs (s, mean ± SEM) in response to a defined radiant heat stimulus. Left: time course. On day 7 after CCI surgery, HZ166 (▼, ▽, 16 mg / kg body weight) or vehicle (●, ○) were injected i.p. and PWTs were monitored for 3 hours after injection. n = 6 mice per group. Right: statistical analysis. AUC (g * h, mean ± SEM). **, $P < 0.01$ (unpaired *t*-test).

Involvement of the BDZ-binding site

To verify that the antihyperalgesic effects of HZ166 came from its interaction with the BDZ-site of $GABA_A$ receptors, we tested the effect of flumazenil, a competitive BDZ-site antagonist (Fig. 3D). Flumazenil (10 mg / kg, i.p., compare (Knabl *et al.*, 2009)) almost completely reversed the antihyperalgesic effect of HZ166 (16 mg / kg). The AUC calculated in flumazenil treated mice (0.54 ± 0.25 g * h; n = 6) was different from that of vehicle treated mice (1.90 ± 0.29 g * h; n = 6; $P < 0.01$, unpaired t-test).

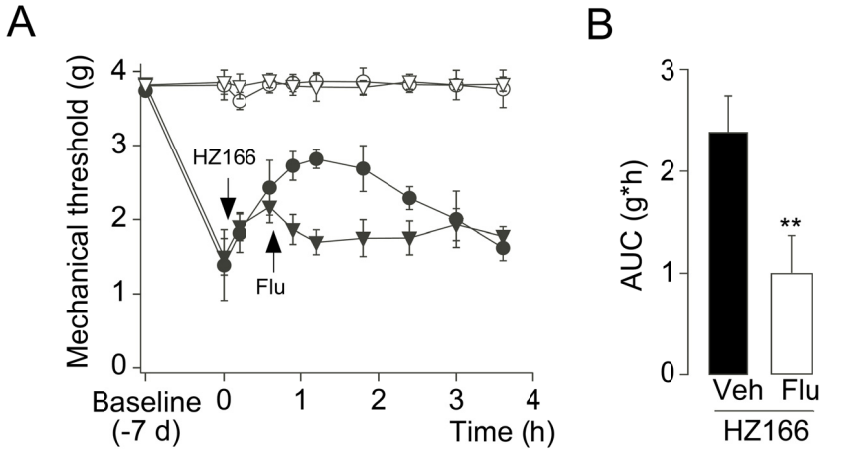

Fig. 3 Reversal of HZ166-induced antihyperalgesia by the BDZ-site antagonist flumazenil. (**A**) PWTs (g, mean ± SEM) in response to von Frey filament stimulation were monitored before and 7 days after nerve ligation. On day 7, HZ166 (16 mg / kg body weight) was injected i.p.. 45 min later flumazenil (Flu, ▼, ▽ 10 mg / kg body weight) or vehicle (Veh, ●, ○) were injected i.p. PWTs were monitored for 3 hours after injection. n = 6 mice per group. (**B**) statistical analysis. AUC (g * h, mean ± SEM) was calculated for the time interval between administration of flumazenil or vehicle (45 min) and the end of the experiment (4 hours). **, $P < 0.01$ (unpaired *t*-test).

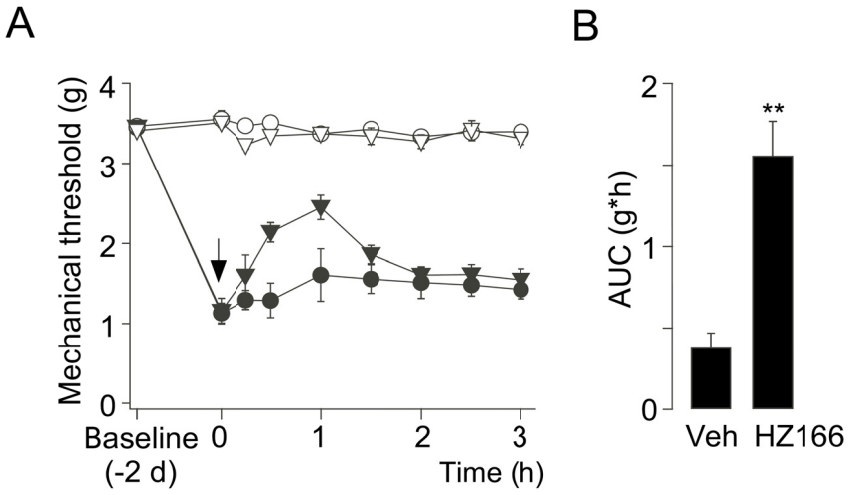

Fig. 4 Antihyperalgesic effects of HZ166 in the zymosan A model of inflammatory pain. Inflammatory hyperalgesia induced by subcutaneous zymosan A injection into the plantar side of the left hindpaw. (**A**) mechanical PWTs (g, mean ± SEM) in response to stimulation with dynamic von Frey filaments were monitored before and 2 days after injection of zymosan A. On day 2, HZ166 (▼, ▽, 16 mg / kg body weight) or vehicle (●, ○) were injected i.p. and PWTs were monitored for 3 hours after HZ166 injection. n = 6 mice per group. (**B**) statistical analysis. AUC (g * h, mean ± SEM). **, $P < 0.01$ (unpaired *t*-test).

Antihyperalgesic effect of HZ166 in the zymosan A model of inflammatory pain

Zymosan A injected subcutaneously into the plantar side of the left hindpaw at a dose of 0.06 mg in 20 µl induced a local inflammatory reaction accompanied by swelling of the paw

and thermal and mechanical sensitization. 48 hours after injection of zymosan A, HZ166 (16 mg / kg) or vehicle were given i.p. and mechanical PWTs were assessed for 3 hours (Fig. 3). The AUC determined in HZ166 treated mice (1.55 ± 0.21 g * h; n = 6 mice) was significantly different from that of vehicle treated mice (0.38 ± 0.08 g * h; n = 6; $P < 0.01$, unpaired t-test).

Comparison of the antihyperalgesic effects of HZ166 and gabapentin

We next determined the antihyperalgesic effects of gabapentin, a drug routinely used in the clinical treatment of neuropathic pain, in the CCI model to compare them with those of HZ166 (Fig. 5). Gabapentin was injected i.p. in mice at doses of 3, 10, 30 and 90 mg / kg and mechanical PWTs were measured for 3 hours after application. Gabapentin caused a dose-dependent reversal of mechanical hypersensitivity. Both compounds evoked antihyperalgesia with similar potencies (ED50 = 6.2 ± 0.8 and 4.3 ± 0.4, for gabapentin and HZ166, respectively) but the maximum antihyperalgesic effect (Emax), which was reached in the case of gabapentin only with sedative doses, was higher for gabapentin (Emax = 82.7 ± 3.5 and 59.7 ± 1.5 for gabapentin and HZ166, respectively).

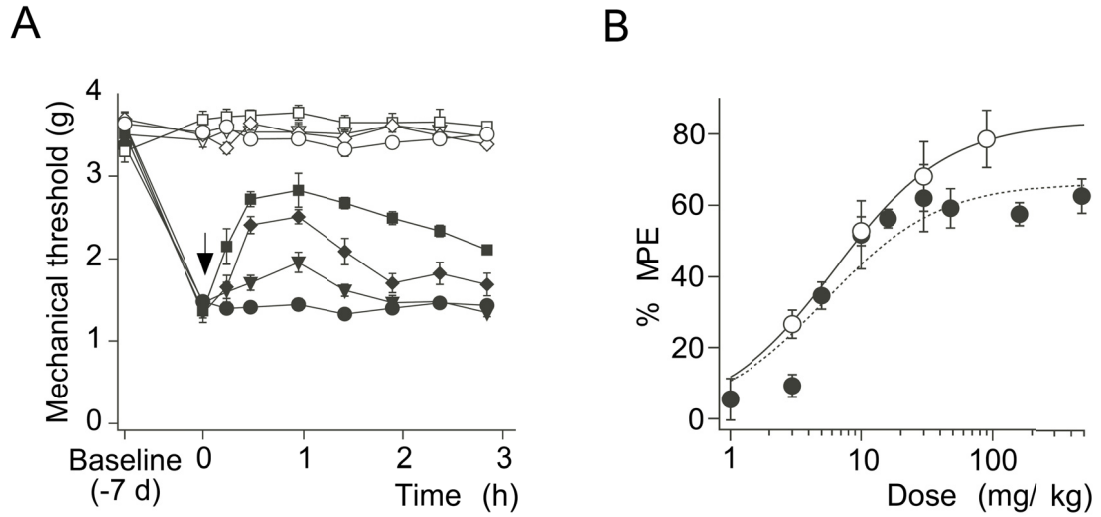

Fig. 5 Effect of gabapentin on mechanical sensitization after chronic constriction injury. (**A**) PWTs (g, mean ± SEM) in response to von Frey filament stimulation were monitored before and 7 days after CCI surgery. On day 7, gabapentin (3 [▼, ▽], 10 [◆, ◇] or 30 [■, □] mg / kg body weight) or vehicle (●, ○) were injected i.p. and PWTs were monitored for 3 hours after gabapentin injection. n = 6 mice per group. (**B**) Per cent maximum possible effect of gabapentin on mechanical sensitization after CCI. For comparison the data for HZ166 (same as in Fig. 2B) are displayed again.

Motor coordination and locomotor activity

We used the rotarod test to assess possible drug-induced changes in motor performance (Fig. 6A). Doses ≤ 160 mg / kg (HZ166) and ≤ 30 mg / kg (gabapentin) did not interfere with rotarod performance. The propensity of HZ166 and gabapentin to cause sedation was evaluated in individual automated circular enclosures. (Fig. 6B). At doses of 16 and 48 mg / kg, which already yielded maximum antihyperalgesic effects, HZ166 did not significantly impair motor activity. Significant reduction of activity was observed only at higher doses (100 and 160 mg / kg). In case of gabapentin a significant reduction in locomotor activity was found at doses ≥ 30 mg / kg.

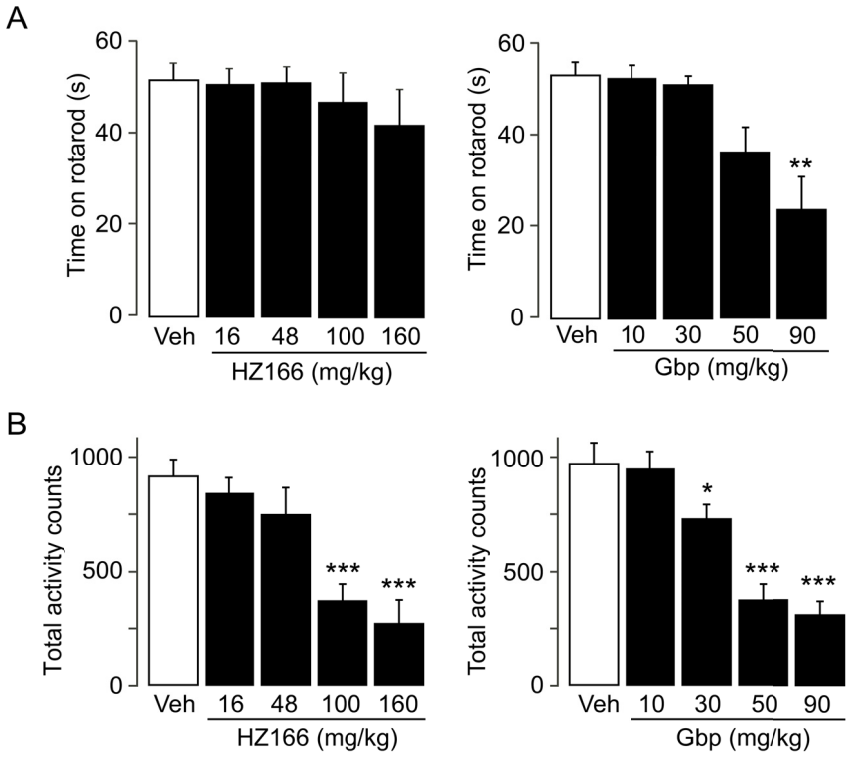

Fig. 6 Effect of HZ166 and gabapentin on motor coordination and motor activity. (**A**) Rotarod test. Left: total time spent on rotarod (mean ± SEM) measured 60 min after the injection of HZ166 (16, 48, 100 and 160 mg /kg body weight, i.p.) or vehicle. n = 10 mice per group. Right: same as left but gabapentin (10, 30, 50 and 90 mg / kg body weight, i.p.) or vehicle. ANOVA followed by Scheffe's post hoc test, F (4, 45) = 7.29; **, $P < 0.01$, significantly different from vehicle-treated controls. (**B**) Motor activity. Left: total activity counts (mean ± SEM) measured for 60 min starting 60 min after the injection of HZ166 (16, 48, 100 and 160 mg /kg body weight, i.p.) or vehicle. n = 10 mice per group. ANOVA followed by Scheffe's post hoc test, F (4, 45) = 11.39; ***, $P < 0.001$, significantly different from vehicle-treated controls. Right: same as left but gabapentin (10, 30, 50 and 90 mg / kg body weight, i.p.) or vehicle. ANOVA followed by Scheffe's post hoc test, F (4, 45) = 22.31; *, $P < 0.05$; ***, $P < 0.001$, significantly different from vehicle-treated controls.

Tolerance development

Long-term administration of BDZs is often associated with a progressive loss of therapeutic activity (tolerance development). Here, we investigated the liability of HZ166 to tolerance development (Fig. 7). Mice were chronically treated with HZ166 at a dose of 16 mg / kg once daily or with vehicle for 9 days starting from day 7 after CCI surgery. After 10 days of chronic drug or vehicle treatment, mechanical sensitivity was measured in both groups after administration of HZ166 to compare its analgesic activity in drug-naïve mice and in mice chronically exposed to HZ166. HZ166 exerted virtually the same analgesic activity in both groups. Measurements of mechanical sensitivity were also performed at day 3 and 5 during chronic treatment to monitor the antihyperalgesic activity of HZ166 over time. At neither one of these time points any signs of reduced antihyperalgesic activity were found.

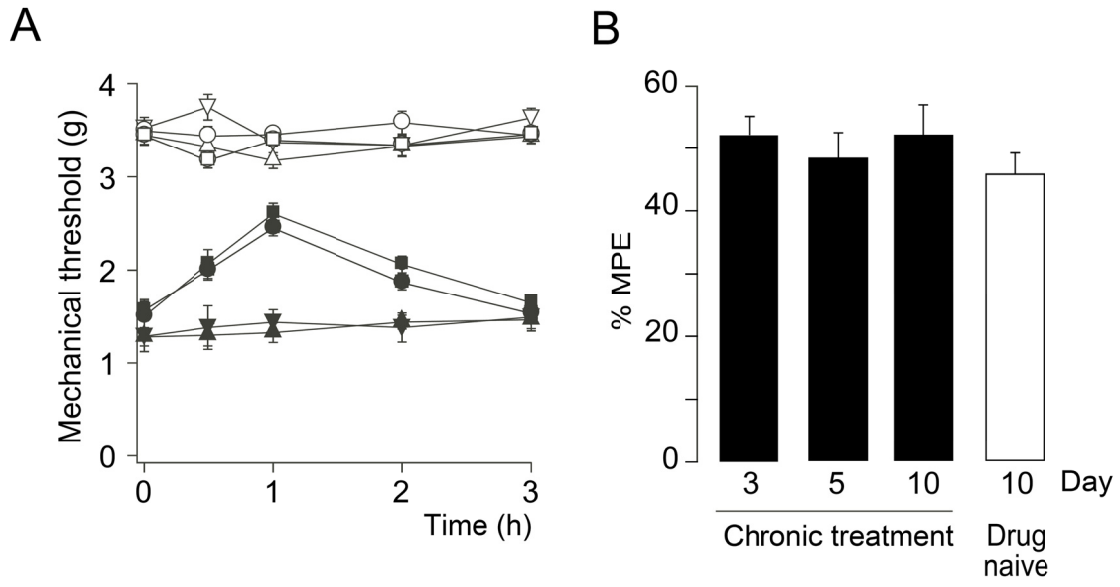

Fig. 7 Lack of tolerance development. (**A**) PWTs (g, mean ± SEM) in response to von Frey filament stimulation were assessed in mice 17 days after CCI surgery. Mice had undergone 10 days of chronic i.p. treatment with either HZ166 (16 mg / kg body weight) or vehicle, and were treated acutely on day 17 with either HZ166 (16 mg / kg body weight) or vehicle. 4 groups of mice were analyzed (chronic vehicle / acute vehicle [▼, ▽], chronic vehicle / acute HZ166 [■, □], chronic HZ166 / acute HZ166 [●, ○], chronic HZ166 / acute vehicle [▲,△]). PWTs were monitored for 3 hours after drug injection. Filled symbols, ipsilateral paw; open symbols, contralateral paw. n = 6 mice per group. (**B**) Per cent maximum possible effect of HZ166 after 3, 5 and 10 days of chronic treatment with HZ166 (black bars) and in mice chronically treated with vehicle for 10 days (white bar). n = 6 mice per group. ANOVA followed by Scheffe's post hoc test, F (3, 20) = 1.50.

Discussion

Our previous studies in $GABA_A$ receptor point-mutated mice have shown that spinal α2- and/or α3-$GABA_A$ receptors mediate most of the anti-hyperalgesic activity observed with spinally administered diazepam, while sedative α1-$GABA_A$ receptors do not contribute. After systemic treatment with diazepam, a strong antihyperalgesic action was retained in α1-$GABA_A$ receptor point-mutated mice suggesting that non-sedative (α1 sparing) BDZ-site agonists should exert pronounced antihyperalgesic effects (Knabl *et al.*, 2009). Such pharmacological data have hitherto largely been missing in mice, which precluded a comparison of genetic and pharmacological data within the same species. To address this issue, we have now used HZ166 a novel non-sedative BDZ-site partial agonist. As shown in this study, HZ166 rapidly penetrates into the CNS and possesses sufficiently long half life to make it a suitable compound for behavioral testing in mice. The present data confirmed that the antihyperalgesic actions of HZ166 correlate well with its pharmacokinetic profile in the CNS. As shown previously, HZ166 possesses the highest intrinsic activity at α3 and α2 $GABA_A$ receptors and much less intrinsic activity at α1 and α5 $GABA_A$ receptors (Rivas *et al.*, 2009). It needs to be added here that diazepam also possesses higher intrinsic activity at α3 and α2 than at α1 $GABA_A$ receptors. However, the selectivity coefficient (activity at α2 versus activity at α1-$GABA_A$ receptors) is 1.87 for HZ166 and 1.47 for diazepam (calculated from the potentiation of $GABA_A$ receptor currents [at EC_3 of GABA] (Rivas *et al.*, 2009). The present study shows in addition that HZ166 also exhibits significant differences in binding affinities to $GABA_A$ αx/β3/γ2 receptors with a rank order of α5 > α3 > α2 > α1. Affinity to α2-$GABA_A$ receptors was about 40% higher than that to α1-$GABA_A$ receptors. This differential affinity at recombinant $GABA_A$ receptor subtypes most likely translates into the *in vivo* situation where HZ166 exhibited different binding affinities to brain and spinal cord membranes. The improved intrinsic activity profile together with the higher affinity at α2- versus α1-$GABA_A$ receptors probably underlies the better separation of antihyperalgesia from sedation by HZ166. In fact, in a previous publication (Knabl *et al.*, 2009) we have shown that diazepam-induced analgesia and sedation occurred with similar dose-dependencies, while with HZ166 estimated ED_{50} values for antihyperalgesia (5.3 mg/kg) and sedation (97 mg/kg; compare

Fig. 6) differed by a factor of almost 20. Furthermore, in the rotarod test we did not measure any significant impairment at doses up to 160mg/kg, which was more than 10 times the dose yielding maximal antihyperalgesia.

Our behavioral experiments hence indeed demonstrate that HZ166 possesses pronounced activity against thermal and mechanical hyperalgesia in models of inflammatory and neuropathic pain at doses devoid of sedation or motor impairment. It is still not clear whether this improved side effect profile translates to humans. The clinical development of MK409, another subtype selective agent which was non-sedating in preclinical models, was stopped because of sedative effects in man. These findings may suggest that humans could be more susceptible to sedation than rodents (Atack *et al.*, 2010). However, available evidence for HZ166 indicates that it does not cause sedation in primates at anxiolytic doses despite some retained activity at α1-$GABA_A$ receptors (Fischer *et al.*, 2010).

Importantly, HZ166-induced antihyperalgesia was reversed by flumazenil, a competitive BDZ-site antagonist, indicating that these effects were specifically mediated by $GABA_A$ receptors. These data are in line with previous results obtained in rats by our group with L-838,417 (Knabl *et al.*, 2008) or by others with NS11394 (Munro *et al.*, 2008), which is another subtype-selective BDZ-site ligand (Mirza *et al.*, 2008). Together, they indicate that profound antihyperalgesia can be obtained in different rodent species with non-sedative BDZ-site ligands in various pain models.

Similar to what we and other have seen with intrathecal injections of diazepam in mice (Knabl *et al.*, 2009) or with systemic administration of subtype-selective agents in rats (Knabl *et al.*, 2008; Munro *et al.*, 2008), HZ166 did not change responses of non-inflamed or uninjured paws confirming that a facilitation of $GABA_A$ receptor-mediated inhibition at the spinal cord level produces anti-hyperalgesia rather than general analgesia.

In order to compare the antihyperalgesic efficacy of HZ166 to that of drugs already established in the treatment of neuropathic pain, we compared the effects of HZ166 with those of gabapentin, an anticonvulsant drug which is widely used in the treatment of different forms of neuropathic pain. Its clinical effectiveness has been described in a variety of pain syndromes, including painful diabetic neuropathy, postherpetic neuralgia and phantom limb pain (Jensen *et al.*, 2009). Gabapentin is generally well tolerated but sedation

and dizziness are reported adverse effects. Here, we found that at non-sedative doses, the antihyperalgesic activities of gabapentin and HZ166 were similar. Nevertheless, the maximum possible antihyperalgesic effect of gabapentin was higher, but only at doses, which significantly reduced motor activity and which were thus considered to be sedative. Gabapentin also caused a significant impairment of motor performance in the rotarod test at a dose of 90 mg / kg, while HZ166 did not do so even at doses up to 160 mg / kg.

Apart from sedation, major side effects of classical BDZs as well as of most centrally acting analgesics include tolerance development and addiction. We have previously reported that analgesic effect of L-838,417 did not decline during repeated administration (once per day for nine days), while complete tolerance developed during the same time period against morphine-induced analgesia (Knabl *et al.*, 2008). Here, we found again no loss of antihyperalgesic activity of HZ166 during a 9-day treatment period in neuropathic mice. Although the lack of tolerance development likely results from reduced activity at α1-$GABA_A$ receptors (Mirza *et al.*, 2006), pharmacokinetic properties (time of exposure of the receptors to the drug) and a generally lower intrinsic activity (as compared to diazepam) may also contribute (Licata *et al.*, 2008).

We did not test for potential addictive properties of HZ166, but available pharmacological evidence again suggests that α1 sparing agonists should be devoid of addictive properties (Ator *et al.*, 2010). A critical role of α1 $GABA_A$ receptors in re-enforcing properties of BDZs has also been demonstrated in a recent neurobiological study (Tan *et al.*, 2010), which used mice carrying point-mutated (diazepam-insensitive) $GABA_A$ receptor subunits.

In summary, our findings suggest that future subtype-selective (α1 sparing) BDZs may constitute a novel approach to the treatment of chronic pain. Drug companies have already tried to identify and develop α1 sparing compounds in the sake for novel non-sedative anxiolytics (Atack, 2005). This strategy has yielded only rather limited success so far, partly because most known subtype-selective BDZ-site agonists differ primarily in their intrinsic activity at $GABA_A$ receptor subtypes while traditional drug screening relied on differences in affinity. The recent advent of novel technologies in electrophysiology which allow high throughput screening of agents acting on ion channels (Dunlop *et al.*, 2008) should significantly facilitate the identification of such compounds.

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GENERAL DISCUSSION

Evidence from several groups supports the concept that a loss of inhibitory control of nociceptive neurons contributes to the pathology of different pain states (Zeilhofer *et al.*, 2012). In this thesis, I have explored the possibility that diminished synaptic inhibition occurs also at the level of the brainstem and contributes to pain during migraine attacks. Two findings suggest that PGE_2-mediated inhibition of glycine receptors (Harvey *et al.*, 2004) might be a particularly relevant mechanism leading to central sensitization in migraine: first, migraine headache and migraine associated allodynia respond well to cyclooxygenase inhibitors (Jakubowski *et al.*, 2005) and, second, pharmacological inhibition of glycine receptors in the spinal cord or the brainstem evokes profound allodynia (Miraucourt *et al.*, 2007; Zeilhofer, 2005). To analyze the contribution of EP2/GlyRα3 pathway, I first used *cacna1a* (R192Q) mice, which have been reported to develop spontaneous headache episodes (Langford *et al.*, 2010), and compared them with double mutant mice, carrying both the *cacna1a* (R192Q) mutation and a knock-out allele of either the EP2 receptor or the Glyα3 receptor. Although susceptibility to chemically evoked CSD was increased in these mice, I did not find evidence of an increased photophobic behavior or an increased *c*-fos activation in the TNC in freely moving *cacna1a* (R192Q) mice. It was therefore not surprising that the phenotype of the single mutant *cacna1a* (R192Q) mice was not different from that of double mutant mice. To circumvent this problem, I analyzed chemically evoked CSD in $EP2^{-/-}$ and in $GlyR\alpha3^{-/-}$ mice. While $GlyR\alpha3^{-/-}$ mice displayed a similar susceptibility to KCl-induced CSD as wild-type littermates, CSD thresholds of $EP2^{-/-}$ mice were similar to those of *cacna1a* (R192Q) mice. Both $EP2^{-/-}$ and in $GlyR\alpha3^{-/-}$ mice were not photophobic and did not present an increased TNC activation after CSD compared to wild-type mice. In summary, analyzing electrophysiological and behavioral correlates of migraine and neurochemical markers of trigeminal activation, I did not find evidence for a major contribution of the EP2/GlyRα3 pathway. These negative findings do however not exclude that disinhibition still makes a major contribution of migraine. Disinhibition in the brainstem may occur through mechanisms different from the EP2/GlyRa3 pathway, and/or at other sites, such as higher brain areas including the thalamus. Interestingly, recent studies have highlighted the

importance of thalamic processing of trigeminovascular nociceptive transmission in neurobiological mechanisms contributing to extra facial allodynia and photophobia (Noseda *et al.*, 2010). Third-order trigeminovascular neurons have their cell bodies in the posterior dorsal thalamus and they project to different area of the cortex involved in various functions possibly explaining the diversity of neurological symptoms associated with migraine headache (Noseda *et al.*, 2011). These third-order thalamic neurons have been shown to be sensitized after dural stimulation with an inflammatory soup and to mediate the spread of allodynia and hyperalgesia outside the original site of migraine headache (extracephalic) (Burstein *et al.*, 2010). Moreover, it has been proposed that a distinct retinal pathway modulating thalamocortical neurons is involved in the "photoregulation" (Noseda *et al.*, 2010) of migraine headache. Some of the thalamocortical neurons in fact increase their activity in response to the photic signals they receive from retinal ganglion cells, resulting in exacerbation of headache perception.

Apart from a loss of inhibition, increase excitation can also underlie an excitation/inhibition imbalance. In this context it is interesting to note that the *cacna1a* (P/Q type calcium channel) R192Q mutation results in increased excitatory synaptic transmission due to an increased release of glutamate but does not affect inhibitory GABAergic neurotransmission (Tottene *et al.*, 2009). These findings suggest that FHM1 mutations alter the neuronal circuits that modulate the balance between excitation and inhibition. This disrupted regulation of excitation/inhibition may account for an abnormal regulation of cortical function and for the episodic vulnerability to CSD occurrence in migraine.

Since P/Q type calcium channels are widely expressed in the CNS, including different cortical regions, trigeminal ganglion, spinal trigeminal nucleus, spinal cord and brainstem (Craig *et al.*, 1998) the imbalance between excitation and inhibition could occur at different levels in the CNS. After all a rational therapeutic approach could therefore be a facilitation of synaptic inhibition (Zeilhofer *et al.*, 2012). Here, we have shown that the pharmacological facilitation of GABAergic inhibition with HZ166, a new partial BDZ-site agonist with preferential activity at α2- and α3$GABA_A$ receptors, reverses signs of neuropathic and inflammatory pain. The observed antihyperalgesic effect most likely comes mainly from the modulation of α2 containing $GABA_A$ receptors located in the dorsal horn of the spinal cord.

TNC is considered to be the site of the second orders neurons of the-nociceptive pathways of the craniofacial region. Notably, immunoreactivity versus both α2 and α3-containing $GABA_A$ receptors have been reported in the TNC of rats (Fritschy *et al.*, 1995; Pirker *et al.*, 2000) and in post-mortem human tissue (Waldvogel *et al.*, 2010). According to the morphological and functional similarities with the dorsal horn of the spinal cord (Bereiter *et al.*, 2000), it is plausible that a facilitation of synaptic inhibition by targeting specific $GABA_A$ receptor subtypes could be beneficial at the level of the brainstem for migraine management. On the other hand, if the impaired balance between excitation and inhibition took place and played a prominent role at the cortical level, it might be more difficult to increase inhibition without causing sedation, since α1-containing $GABA_A$ receptors are the most abundant $GABA_A$ receptors in the cortex (Fritschy *et al.*, 1995). Future experiments investigating the effect of HZ166 on trigeminal sensitization will provide new insight into the usefulness of this pharmacological strategy in the treatment of migraine.

The results of this thesis also indicate that the available genetic and pharmacological models of migraine in rodents are still not satisfying. While the translational value of the pharmacological migraine models is unknown, one should expect that a genetic migraine model would better resemble the human situation (Olesen *et al.*, 2012b). The *cacna1a* (R192Q) mouse is at present by far the best-characterized genetic mouse model of migraine, yet even homozygous *cacna1a* (R192Q) mice only show modest phenotypes in behavioral tests. The only well-documented phenotype described by others is an alteration in facial expression possibly indicating spontaneous discomfort in these mice (Langford *et al.*, 2010). Increased head grooming and photophobia in a modified elevated plus maze test have in addition been reported but so far in abstract form only (Chanda *et al.*, 2008). This absence of a strong behavioral phenotype is surprising given that this mutation causes a particularly strong disease in humans (Ducros *et al.*, 2001). The absence of an obvious strong behavioral phenotype does not necessarily mean that spontaneous migraine attacks do not occur in these mice, but might rather indicate a lack of suitable readouts of migraine attacks in mice. However, neither the Neurologger experiments nor the TNC c-*fos* stainings revealed clear signs of CSD events or of trigeminal sensitization suggesting the mere absence of spontaneous migraine attacks in these mice. Preclinical migraine research would definitely

benefit from the development of more sensitive methods to examine migraine headaches in animal models.

In the absence of suitable behavioral readout of migraine headaches in rodents, electrophysiological correlates of aura and neurochemical markers of trigeminal sensitization are frequently used in rodent migraine studies. Their suitability and in particular the relation between the two is however also a matter of controversial debate. We and other research groups have used dural application of KCl to induce CSD in mice (paper 2, (Ayata *et al.*, 2006; Brennan *et al.*, 2007; Moskowitz *et al.*, 1993) and to study subsequent TGVS activation. Few years after Moskowitz's report on the activation of the TGVS after KCl-induced CSDs (Moskowitz *et al.*, 1993), Ingvardsen and colleagues proposed that the observed c-fos activation in the TNC correlated with the number of KCl application rather than with the number CSDs evoked (Ingvardsen *et al.*, 1997) suggesting that TNC activation might originate directly from a KCl-induced activation of primary meningeal nociceptors and would not require CSD. Furthermore, another study showed that KCl injection into the cortex of freely moving rats elicited cutaneous allodynia and increased *c-fos* staining in the TNC without provoking CSD events, and that mechanical stimulation of the cortex with pinprick did not induce tactile allodynia or TNC activation despite eliciting CSD (Fioravanti *et al.*, 2011). These results are in contrast with recent direct electrophysiological evidence from the group of Burstein (Zhang *et al.*, 2011; Zhang *et al.*, 2010), who demonstrated that induction of CSD by focal stimulation of the rat visual cortex can lead to persistent activation of meningeal nociceptors (Zhang *et al.*, 2010) and can cause increased ongoing activity in central trigeminovascular neurons in the spinal trigeminal nucleus (Zhang *et al.*, 2011). These results provide experimental evidence that a cortical phenomenon can induce sensitization of trigeminovascular neurons and facial allodynia in migraine patients. Further experiments are still needed to determine whether single episodes of evoked CSD are sufficient to activate meningeal nociceptors and trigger migraine pain.

In summary, the results of this thesis highlight that mechanistic studies on migraine would greatly benefit from better rodent models of migraine and in particular from the development of better readouts of migraine attacks in rodents.

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APPENDIX

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
AMPA	2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BDZ	benzodiazepine
BOLD	blood-oxygen-level dependent
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
cAMP	cyclic adenosine monophosphate
CCI	chronic constriction injury
CBF	cerebral blood flow
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
CNS	central nervous system
COX	cyclooxygenase
CSD	cortical spreading depression
D2	dopamine receptor 2
DC	direct current
Dzp/DZP	diazepam
EP2	prostaglandin E2 receptor
EEG	electroencephalography
FHM	familial hemiplegic migraine
GABA	γ-aminobutyric acid
$GABA_A$	γ -aminobutyric acid receptor type A
$GABA_B$	γ -aminobutyric acid receptor type B
GlyRα3	glycine receptor α3 subtype
GPCR	G protein coupled receptor
GTN	glyceryl trinitrate
IASP	International Association for the Study of Pain
IB4	isolectin IB4
i.p.	intraperitoneal
i.t.	intrathecal
KCC2	K^+-Cl^- cotransporter type 2
LC	locus coeruleus
LTP	long-term potentiation
MA	migraine with aura
MO	migraine without aura
MOH	medication overuse headache

MEG	magnetoencephalography
MRI	magnetic resonance imaging
NGF	nerve growth factor
NKCC1	Na^+-K^+-$2Cl^-$ cotransporter type 1
NMDA	*N*-methyl-*D*-aspartic acid
NSAID	non-steroidal anti-inflammatory drug
PAG	periaqueductal gray
PET	positron emission tomography
PGE_2	prostaglandin E2
PKA	protein kinase A
RVM	rostral ventromedial medulla
SC	spinal cord
SP	substance P
SRT	spinoreticular tract
TCC	trigeminocervical complex
TGVS	trigeminovascular system
TNC	trigeminal nucleus caudalis
TNF-α	tumor necrosis factor α
TRESK	TWIK-related spinal cord potassium channel
trkB	tyrosine kinase receptor B
TTX	tetrodotoxin
SD	standard deviation
SEM	standard error of the mean
SWA	slow wave activity
VGCC	voltage-gated calcium channel
Veh	vehicle

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2005-2007	Master Degree in Medical, Molecular and Cellular Biotechnology (Research and Development in Pharmaceutical Biotechnology). University "Vita-Salute San Raffaele", Milano. 110/110 cum laude
2006-2007	Master Thesis c/o Schering Plough Research Institute (SPRI, Milano). Title of the thesis: "*Contribution of neuroinflammatory processes to the maintenance of neuropathic pain: role of microglia and cannabinoid CB2 receptor"*
2008	Employed as PhD student at the University of Zurich since April 2008, Institute of Pharmacology and Toxicology, Neuropharmacology group, Prof. Hanns Ulrich Zeilhofer.

Positions

2006-2007	Internship c/o Schering Plough Research Institute (SPRI, Milano).
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Poster Presentations

"Central Pain Sensitization in Migraine", ***ZNZ PhD Retreat***, Valens, 23th May 2009.

"Characterization of HZ166, a Novel $GABA_A$ Receptor Subtype-Selective Benzodiazepine Site Ligand, in Mouse Pain Models", ***Pharma Poster Day***, Zürich, 10th July 2009.

"Characterization of HZ166, a Novel $GABA_A$ Receptor Subtype-Selective Benzodiazepine Site Ligand, in Mouse Pain Models", ***ZNZ symposium***, Zürich, 11th September 2009.

"A Novel $GABA_A$ Receptor Subtype-Selective Benzodiazepine Site Agonist Is Analgesic in Mouse Pain Models", ***FENS meeting***, Amsterdam, 3-7th July 2010.

"EEG data logging with the NeuroLogger® system: spontaneously occurring electrophysiological correlates of migraine in mutant mice", ***Measuring Behavior,*** Eindhoven, 24-27th August 2010.

"Characterization of HZ166, a Novel $GABA_A$ Receptor Subtype-Selective Benzodiazepine Site Ligand, in Mouse Pain Models", ***IASP meeting***, Montreal, 29th August- 2nd September 2010.

"Neuronal Activity in the Neocortex of Migraine Mutant Mice Recorded with the NeuroLogger System" ***NCCR Neural Plasticity and Repair Symposium,*** Ittingen, 4- 5th February 2011.

"HZ166, a Novel $GABA_A$ Receptor Subtype-Selective Benzodiazepine Site Ligand, Is Antihyperalgesic in Mouse Models of Inflammatory and Neuropathic Pain" ***SSPT meeting***, Zürich, 27-28th April 2011

"HZ166, a Novel $GABA_A$ Receptor Subtype-Selective Benzodiazepine Site Ligand, Is Antihyperalgesic in Mouse Models of Inflammatory and Neuropathic Pain", ***Pharma Poster Day***, Zürich, 6th June 2011.

"HZ166, a novel non-sedative $GABA_A$ receptor benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain", ***SFN meeting***, Washington, 12-16th November 2011

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Zeilhofer H U, Möhler H, Di Lio A. (2009). GABAergic analgesia: new insights from mutant mice and subtype-selective agonists. *Trends in Pharmacological Sciences*. **8**:397-402.

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Acknowledgements

The present thesis was carried out at the Institute of Pharmacology and Toxicology of the University of Zurich in the Neuropharmacology group of Prof. Hanns Ulrich Zeilhofer.

I wish to express my sincere gratitude to Uli, who created the conditions under which this work was possible, for his encouragement, for sharing his knowledge through stimulating discussions and for making continuous efforts to improve my written and oral skills. I particularly appreciated his patience in correcting my proverbial Italian-English without getting tired of it.

I would like to thank Prof. Esther Stoeckli and Prof. Jean-Marc Fritschy for their invaluable time spent as members of my Thesis Committee in these four years. Jean-Marc deserves an additional thank for his morphological "consulting", antibodies supply and for his uncommon scientific and unscientific support.

I spent 4 wonderful years in the Neuropharmacology group thanks to many good colleagues who created a daily stimulating and vibrating research environment.
Thanks Katrin, for being always available to listen to my problems with bills, credit cards and for having the patience to listen to my incorrect German.
Thanks Louis, for your technical support with mouse genotyping, without you all my animal experiments would not have taken place.
Thanks Dimi, for supporting my initial steps into the world of CSD recordings and Neurologger.
Thanks Olivier, for helping me with data interpretation and for all the discussion we had on the obscure world of migraine.
Thanks Dietmar, for teaching me biochemical experiments and helping me performing important experiments for the 3rd project.
Thanks Roland, for your crucial support in the 2nd project, help, discussion and time spent to find a sense to NL recordings and to Peter and Irene for their precious suggestions.

Thanks Florence, for your support with some of the behavioral experiment in the 3rd project and for discussing the data with me.

Thanks Corina, for your time spent working with me as a Master Student. In the 6 months we have performed experiments together I learnt more than I had expected and I found a new strong motivation for my work.

Thanks Simeon, for your contribution as a Medical Doctor to the post-operative pain project. It has been a positive challenge to help you during this time.

Thanks Edmund, for improving the English of this thesis.

Thanks to Robert and Torbjörn, previous PhD students in the lab who have welcomed me 4 years ago and helped me to find a place in the group.

Thanks Khaled and Patrick, for the great time we spent together, as colleagues but especially as friends.

Thanks to Willy, Stefanie, Isa, Jolly, Pradeep, Salim, Ilja, Thomas, Helge, Edmund, Hendrik, Sabine, Karen, Caroline, Mario, Marisa, Valeria, Giannina, Maya, Cagla, Anita, Laetitia (and for all the others I forgot).

I always wonder how my life in the lab would have been without Ako and Gonzalo, who became indissolubly part of my life, wherever they are now and wherever they will be in the future. Their support was one of a kind. Grazie Ako, Gracias Gonzi!

Thanks to all my present and previous colleagues at the Institute, in particular Sonja, Corinne, Irene, Francine, Tina, Sandra, Amrita, Himanish and Tareixa.

A special big thank to Charlie, who inspired my decision to learn German and who provided important help for the behavioral equipment.

Thanks Harald, for being always available for building up electrodes for me.

A special thanks to all the animal caretakers, Kofi, Carmen, Edward, Ana, Conny, Dennis and Boateng. Your work was absolutely important for the success of my experiments.

Thanks to the old Italian mafia group: Ako, Novella, Maria Luisa, Michela and Alessia for the wonderful unforgettable moments we spent in these years.

Thanks to all mice that made my thesis possible.

In Zürich I met many friends and it is extremely difficult for me to say thanks to everyone. I'm convinced that all of you know how important you have been and you will be in my life. However I would like to express my gratitude to some special people who rendered this experience in a class by itself, Ako, Novella, Karelia, Carmela, I Wups! (Daniele, Simone, Andrea, Matteo), Serena, Ludovica, Gaia, Marina, Lea, Rachele, Odete, Lucia, Ilaria, Carolina, Marco, Byron, Mary and many others.

Grazie mamma e papà, il vostro esempio, il vostro appoggio e la vostra comprensione hanno reso questa mia avventura zurighese ancora piu' eccezionale.

Grazie Gioi, sei la sorella che tutti dovrebbero avere, la tua presenza costante e il tuo sostegno, anche a distanza, sono fondamentali nella mia vita.

Grazie a tutti i miei zii e alle mie fantastiche cusins.

Grazie a tutti gli amici lontani e insostituibili. In particolare grazie Raffi, Laura, Raffi, Eli, Fede, Dani, Rossi, Giulia&Giulia, Mari, Nico, Gianluca, Dario.

Infine grazie a chi da lassù è spettatore silenzioso di ogni traguardo della mia vita.